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Regulation of IL-7 Receptor (CD 127) Expression on Circulating CD8+ T Cells in HIV Infection and Its Role in Cytotoxicity

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Regulation of IL-7 Receptor (CD127) Expression on Circulating $CD8^+$ T Cells in HIV Infection and its Role in Cytotoxicity

By

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THESIS

Submitted to the School of Graduate Studies in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

Department of Biochemistry, Microbiology & Immunology
Faculty of Medicine
University of Ottawa

Your file Votre référence
ISBN: 978-0-494-59503-9
Our file Notre référence
ISBN: 978-0-494-59503-9

ABSTRACT

The progressive breakdown of cell mediated immunity is a feature in the pathology of HIV disease. Although not lost from the circulation, with disease progression cytotoxic T lymphocytes (CTL) are less able to contain numerous pathogens including HIV itself. Interleukin (IL)-7 and its signalling via the IL-7 receptor (CD127) is essential for optimal CTL activity. Our demonstration that fewer $CD8^+$ T cells from HIV^+ patients express CD127 as compared to healthy individuals, implicates a role for the regulation of CD127 expression in the immunopathogenesis of HIV. Thus, we hypothesize that altered CD127 expression and/or function on $CD8^+$ T cells contributes to impaired cell mediated immunity.

This thesis reports an examination of which host or viral factors may be responsible for the down-regulation of CD127 on $CD8^+$ T cells in HIV infection and the mechanism(s) by which these host and/or viral factors decrease CD127 expression on $CD8^+$ T cells. It also assesses whether HIV down-regulates CD127 expression on $CD8^+$ T cells *in vitro*, and evaluates if the functional capacity of CD127 is altered in HIV infection.

Of all the host and viral factors tested, IL-7, IL-4 and HIV-1tat caused a down-regulation in surface CD127 expression on $CD8^+$ T cells, without affecting CD127 mRNA. IL-7 did not increase internalization of surface CD127, but caused shedding of the receptor. Furthermore, *In vitro* HIV infection of PBMC caused a

down-regulation in surface CD127 expression on $CD8^+$ T cells, without affecting CD127 mRNA, and seems to be caused by a soluble factor produced by the infected PBMCs. Finally, in response to IL-7, STAT5 phosphorylation in $CD8^+CD127^+$ cells from HIV^+ individuals is significantly decreased compared to healthy controls. Proliferation of $CD8^+CD127^+$ cells in response to IL-7 is also significantly impaired. These data demonstrate a dysfunction in the IL-7 receptor system that may be explained by IL-7 specific signal transduction defects.

As the IL-7/IL-7R system and its disruption during HIV infection continue to be intensively studied, one will get a better understanding of HIV immunopathogenesis and the potential to lead to the development of novel immune based therapies will come about.

ACKNOWLEDGEMENTS

I would like to thank Dr. Jonathan Angel for his support, encouragement, and patience during my time in his laboratory. He has given me every opportunity to develop in my academic endeavours and has helped me to become a confident and independent scientist. Throughout the time in his laboratory, I have gained many attributes, which I am grateful for and know will be useful to me all through my career.

I would also like to thank my family and friends for their underlying support and help. I would especially like to thank my husband, Zoran Vranjkovic, and son Adrijan Vranjkovic, for always believing that I could do anything and never letting me give up. Without their encouragement, I would not have accomplished what I have. I would also like to thank Dr. Karen Copeland as well as Dr. Angela Crawley for their support and scientific discussion regarding this project. Furthermore, I would like to thank other members of Dr. Jonathan Angel's laboratory, past and present, for sharing ideas and making my time in the laboratory enjoyable.

At this time, I would like to acknowledge my thesis advisory committee members, Dr. Kathie Wright, Dr. Ashok Kumar and Dr. Subash Sad for their great deal of insight into this project. I would also like to thank CIHR and the Ontario HIV Treatment Network for funding this work.

TABLE OF CONTENTS

ABSTRACT ii

ACKNOWLEDGEMENT iv

TABLE OF CONTENTS v

LIST OF FIGURES xiii

LIST OF ABBREVIATIONS xvii

1 INTRODUCTION 1

1.1 Acquired Immunodeficiency Syndrome: a Worldwide Pandemic 1

1.2 Cytotoxic T Lymphocytes 1

1.3 Cytotoxic T Lymphocytes and HIV 3

1.4 The Interleukin-7/Interleukin-7 Receptor System 5

1.4.1 Expression 5

1.4.2 Signalling 6

1.4.3 Regulation 8

1.5 Interleukin-7: a Cytokine With Central and Peripheral Activity 9

1.5.1 IL-7/IL-7Rα and Lymphopoiesis 9

1.5.2 IL-7/IL-7Rα and Thymopoiesis 10

1.5.3 Role of IL-7/IL-7Rα in the Regulation of Peripheral T Cell Homeostatis....11

1.5.4 Role of IL-7/IL-7Rα in Immune Responses....12

1.6 The Impact of IL-7/IL-7RA Signalling on $CD8^+$ T cells and CTL Activity... 13

1.7 IL-7/IL-7RA system in Human diseases....15

1.7.1 IL-7 in HIV Disease....15

1.7.2 IL-7Rα (CD127) Expression in HIV Disease....17

1.8 IL-7 Immunotherapy for HIV....18

1.9 Rational....21

1.10 Hypothesis....22

1.11 Specific Aims....22

2 MATERIALS AND METHODS....23

2.1 Viral Stocks....23

2.1.1 Preparation and Expansion of HIV-1 Virus Stocks....23

2.1.2 Determination of HIV-1 p24 in Viral Stocks....24

2.1.3 $TCID_{50}$ Determination of Viral Stocks....24

2.2 Preparation of Cells....26

2.2.1 Cell Lines 26

2.2.2 Peripheral Blood Mononuclear Cells (PBMCs) 26

2.2.3 Purified $CD8^+$ T Cells 27

2.3 Cell Culture Conditions for Evaluating the Role of Viral/Host Factors on Surface CD127 Expression 27

2.4 Cell Culture Conditions for Evaluating the Role of *In Vitro* HIV-1 Infection on Surface CD127 Expression 28

2.4.1 In vitro HIV-1 Infection of PBMC and Purified $CD8^+$ T Cells 28

2.4.2 Culturing Conditions 29

2.4.3 Neutralization of IL-7 30

2.4.4 IL-7 and IL-4 ELISA 30

2.5 Statistical Analysis 31

2.6 Flow Cytometric Analysis of Surface CD127 31

2.7 CD127 mRNA Quantitation 32

2.7.1 From Purified $CD8^+$ T Cell Cultures 32

2.7.2 From PBMC Cultures 33

2.7.3 Determination of CD127 mRNA Expression in $CD8^+$ T Cells by Semi-Quantitative PCR 33

2.7.4 Determination of CD127 mRNA Expression in $CD8^+$ T Cells by Real-Time PCR 34

2.8 Immunoflorescence Microscopy for CD127 36

2.8.1 Culturing Conditions 36

2.8.2 Staining for CD127 and CD132 36

2.9 Western Blotting for CD127 37

2.9.1 Culturing Conditions and Harvesting of Supernatants 37

2.9.2 Quantitation of Protein Content in Supernatants 37

2.9.3 SDS-Page 38

2.9.4 Soluble CD127 in Human Plasma Samples 38

2.10 IL-7R Functional Assays 39

2.10.1 CD127 Expression in Whole Blood 39

2.10.2 Preparation of cells 39

2.10.3 CFSE Labelling and T Cell Proliferation Assay 41

2.10.4 Intracellular Phosphorylated STAT5 Expression 41

2.10.5 Expression of CD107a/b 42

2.10.6 Intracellular Bcl-2 Expression 42

2.10.7 Statistical Analysis 43

3 RESULTS 44

3.1 Determining which Host or Viral Factors May be Responsible for the Donwregulation of CD127 Expression Observed in HIV Infection 44

3.1.1 IL-7 Decreases CD127 Expression on $CD8^+$ T Cells in PBMC Cell Cultures 44

3.1.2 IL-7 Decreases CD127 Expression on Purified $CD8^+$ T Cells, Mainly on Naïve $CD8^+$ T Cells 47

3.1.3 IL-4 Decreases CD127 Expression on $CD8^+$ T Cells in PBMC cultures 47

3.1.4 IL-4 Decreases CD127 Expression on Purified $CD8^+$ T Cells, Mainly on Naïve $CD8^+$ T Cells 50

3.1.5 HIV-1 tat Decreases CD127 Expression on Purified $CD8^+$ T Cells 50

3.1.6 Changes in CD127 Expression Are not Due to Mortality 50

3.1.7 HIV-1 gp120, IL-1-β, IL-6, IL-10, IL-13, TGF-β or TNF-α Have no Effect on CD127 Expression 50

3.2 Determining the Mechanism by which Host or Viral Factors Decrease CD127 Expression on $CD8^+$ T Cells 54

3.2.1 CD127 RNA Expression is Unchanged by IL-7, IL-4 and HIV-1 tat 54

3.2.2 IL-7 Does not Increase Cytokine Receptor Internalization 54

3.2.3 IL-7 Induces CD127 Shedding 61

3.2.4 Increased CD127 in the Plasma of HIV$^+$ Individuals Compared to HIV$^-$ Individuals.....65

3.3 Determining if *in Vitro* HIV Infection Results in a Down-regulation of CD127 in CD8$^+$ T Cells 67

3.3.1 In Vitro HIV-1 Infection Decreases CD127 Surface Expression on CD8$^+$ T Cells in PBMC Cultures 67

3.3.2 In Vitro HIV-1 Infection Has no Effect on CD127 Surface Expression in Purified CD8$^+$ T Cell Cultures 67

3.3.3 Soluble Factor Generated by HIV-1 Infection Affects CD127 Surface Expression on CD8$^+$ T Cells.....69

3.3.4 *In vitro* HIV- induced decrease of surface CD127 not due to increased IL-7 or IL-4 production by PBMCs 69

3.3.5 In Vitro HIV-1 Does not Alter CD127 mRNA Levels 73

3.4 Determining if the Functional Capacity of CD127 is Altered in HV Infection 73

3.4.1 Patient Subjects and Study Design 73

3.4.2 Phenotypic Analysis of Patient PBMCs 77

3.4.3 Decreased IL-7-Induced P-STAT5 Activation in HIV-Infected Individuals....77

3.4.4 No Change in IL-7-Induced Bcl-2 Production Between Healthy and HIV-Infected Individuals....80

3.4.5 No Change in IL-7-Induced CD107a/b Expression Between Healthy and HIV-Infected Individuals....84

3.4.6 Impaired IL-7-Induced $CD8^+CD127^+$ T Cells Proliferation in Untreated HIV-Infected Individuals....84

4 DISCUSSION....89

4.1 Determining which host or viral factor(s) may be responsible for the down-regulation of CD127 on $CD8^+$ T cells in HIV infection, and the mechanism thereof....89

4.1.1 Role of Host Factors in the Downregulaiton of CD127 on $CD8^+$ T cells....90

4.1.2 Role of Viral Factors in the Down-regulation of CD127 on $CD8^+$ T cells....97

4.2 To determine if *in vitro* HIV down-regulates CD127 expression on $CD8^+$ T cells....98

4.3 To determine if the functional capacity of CD127 is altered in HIV infection and if this is restored with suppression of viral replication....100

4.3.1 Activation of STAT5 101

4.3.2 Cell Proliferation 104

4.3.3 Expression of Bcl-2 106

4.3.4 Expression of CD107a/b 108

5 CONCLUSION 110

6 REFERENCES 113

7 CURRICULUM VITAE 134

8 CONTRIBUTIONS OF COLLABORATORS 139

LIST OF FIGURES

FIGURE 1: Effect of IL-7 on CD127 expression on $CD8^+$ T cells within PBMC cultures.....45

FIGURE 2: Gating on $CD45RA^+$ cells reflects an accurate evaluation of the expression of CD127 on naïve cells.....46

FIGURE 3: IL-7 decreases surface CD127 expression in purified $CD8^+$ T cell cultures.....48

FIGURE 4: IL-4 decreases surface CD127 expression on $CD8^+$ T cells in PBMC cultures.....49

FIGURE 5: IL-4 decrease surface CD127 expression in purified $CD8^+$ T cell cultures.....51

FIGURE 6: HIV-1 tat decrease surface CD127 expression in purified $CD8^+$ T cell cultures.....52

FIGURE 7: Down-regulation in CD127 expression is not due to cell death.....53

FIGURE 8: IL-1β, IL-6, IL-10 and IL-13 have no effect on the expression of surface CD127 on $CD8^+$ T cells.....55

FIGURE 9: TGF-β, IFN-α and TNF-α have no effect on the expression of surface CD127 on $CD8^+$ T cells.....56

FIGURE 10: HIV-1 gp120 and HIV-1 nef proteins have no effect on the expression of surface CD127 on $CD8^+$ T cells.....57

FIGURE 11: IL-7 has no effect on the expression of mRNA-encoding membrane-bound CD127 mRNA in $CD8^+$ T cells.....58

FIGURE 12: IL-7 does not significantly increase cytoplasmic CD127 in $CD8^+$ T cells.....59

FIGURE 13: HIV-1 tat has no effect on the expression of mRNA-encoding membrane-bound CD127 mRNA in $CD8^+$ T cells.....60

FIGURE 14: IL-4 has no effect on the expression of mRNA-encoding membrane-bound CD127 mRNA in $CD8^+$ T cells.....62

FIGURE 15: Lack of internalization is not due to proteosomal degradation of CD127.....63

FIGURE 16: Analysis of soluble CD127 in $CD8^+$ T cell culture supernatants.. ..64

FIGURE 17: Detection of soluble CD127 in human plasma in health and HIV disease.....66

FIGURE 18: Effect of different strains of HIV-1 on CD127 surface expression on $CD8^+$ T cells in PBMC cultures.....68

FIGURE 19: Effect of different strains of HIV-1 on CD127 surface expression on purified $CD8^+$ T cells.....70

FIGURE 20: Effect of different strains of HIV-1 on CD127 surface expression on purified $CD8^+$ T cells cultured with infected PBMC via a transwell.....71

FIGURE 21: Effect of supernatants from HIV-infected PBMCs on CD127 surface expression on purified $CD8^+$ T cells.....72

FIGURE 22: In vitro HIV-induced decrease in surface CD127 expression on $CD8^+$ T cells in not due to increased IL-4 or IL-7 production.....74

FIGURE 23: Effect of different strains of HIV-1 on CD127 gene expression in $CD8^+$ T cells.....75

FIGURE 24: CD4 count, VL and therapy status of HIV-infected patients.....76

FIGURE 25: CD127 expression levels on $CD8^+$ T cells.....78

FIGURE 26: HIV-infected individuals have decreased IL-7-induced phophorylation of STAT5.....79

FIGURE 27: Correlations between CD127 expression levels or CD4 counts and P-STAT.....81

FIGURE 28: IL-7-induced Bcl-2 expression levels in healthy, untreated and treated HIV-infected individuals.....82

FIGURE 29: HIV-infected individuals on therapy have increased baseline Bcl-2 expression.....83

FIGURE 30: IL-7 induced expression of CD107a and CD107b in healthy, untreated and treated HIV-infected individuals.....85

FIGURE 31: Sub-maximal dose of PHA is required to study the effects of IL-7 on T cell proliferation.....86

FIGURE 32: IL-7-induced proliferation is impaired in HIV-infected individuals...88

LIST OF ABBREVIATIONS

aa	Amino acid
AIDS	Acquired immunodeficiency syndrome
AKT	Protein Kinase B
APC	Antigen presenting cells
BAD	Bcl-2-associated death promoter
BAX	Bcl-2–associated X protein
Bcl	B-cell lymphoma
BSA	Bovine serum albumin
CFSE	Carboxyfluorescein succinimidyl ester
CMV	Cytomegalovirus
CTL	Cytotoxic T cells
DAPI	4,6-Diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
DP	Double positive
EBV	Epstein-barr virus

ELISA	Enzyme-linked immunosorbent assay
FasL	Fas Ligand
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
HAART	Highly active antiretroviral therapy
HIV	Human immunodefiency virus
HRP	Horseradish peroxidise
HSV	Herpes simplex virus
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
JAK	Janus kinase
kDA	Kilo Dalton
LAMP	Lysosome associated membrane protein
LCMV	Lymphocytic Choriomeningitis Virus
MAPK	Mitogen-activated protein kinase
MHC	Major histocompability complex

MLC	Mixed lymphocyte culture
MS	Multiple sclerosis
PBS	Phosphate buffered saline
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PE	Phycoerythrin
PHA	Phytohemaglutinin
PI3K	Phosphatidyl inositol 3 kinase
PIP	phosphatidylinositol bisphosphate
RNA	Ribonucleic acid
RT	Reverse transcription
RSV	Respiratory syncytial virus
SCID	Severe combined immunodeficiency
SIV	Simian immunodeficiency virus
SP	Single positive
STAT	Signal transducer and activator of transcription
$TCID_{50}$	Tissue culture infectivity dose

TCM	T central memory
TCR	T cell receptor
TEM	T effector memory
TGF	Transforming growth factor
Th	T helper
TMB	Tetramethylbenzidine
TN	Triple negative
TNF	Tumour necrosis factor
UNAIDS	United Nations programme on HIV/AIDS

1 INTRODUCTION

1.1 Acquired Immunodeficiency Syndrome: a Worldwide Pandemic

First identified in 1983, the Human Immunodeficiency Virus (HIV) is the etiologic agent of acquired immunodeficiency syndrome (AIDS) [1]. HIV infection causes a progressive degeneration of the immune system, resulting in a decline in $CD4^+$ T cell numbers, defective T cell and macrophage immunological functions, and a deregulation of cytokine production [1-3]. With ongoing accumulation of new infections and longer survival times of HIV-infected individuals, an estimated 30.6-36.1 million persons are living with HIV/AIDS [4]. In the 2007 AIDS Epidemic Update, the Joint United Nations Programme on HIV/AIDS (UNAIDS) and World Health Organization (WHO) state that every day, over 6800 individuals become infected with HIV and over 5700 individuals die from AIDS. The world pandemic of HIV/AIDS has been with us for over 20 years, and has become the greatest infectious disease challenge to public health showing no signs of abatement. For those reasons, the development of much needed immune-based therapies for patients with HIV infection is essential.

1.2 Cytotoxic T Lymphocytes

Demonstrated exhaustively in both murine and human models, $CD8^+$ cytotoxic T lymphocytes (CTLs) are important mediators of adaptive immunity against viral, protozoan, and bacterial pathogens. The CTLs recognize these pathogens by specific interactions between their T cell receptor (TCR) and an 8-10 amino acid processed foreign peptide located in the groove of major

histocompability complex (MHC) I molecules present on the surface of professional antigen-presenting cells (APCs) [5]. Following MHC-mediated recognition of cognate peptide, naïve $CD8^+$ CTLs proliferate and differentiate in response to antigen, and exhibit two general effector functions: 1) target cell killing, and 2) the production of soluble factors, such as cytokines and chemokines.

$CD8^+$ CTLs mediate the killing of target cells via two major pathways: 1) a granule-dependent mechanism that involves perforin and granzymes, and 2) a granule-independent mechanism that involves a ligand–ligand induced cell death (e.g. fas-fasL) [6]. The granule-dependent pathway uses pre-formed lytic granules located within the cytoplasm of the $CD8^+$ CTLs [6]. These lytic granules are membrane-bound secretory lysosomes that contain various proteins, including perforin and granzymes [7]. The protein core of granules is surrounded by a lipid bilayer containing lysosomal associated membrane glycoproteins CD107a (LAMP-1) and CD107b (LAMP-2) [7]. MHC-mediated recognition of cognate peptide and TCR stimulation initiates a polarized mobilization of microtubules that transport the lytic granules towards the immunological synapse formed between the $CD8^+$ CTL and target cells [8]. Once the granules reach the plasma membrane of the CTL, the membranes fuse [7], releasing the granule contents into the immunological synapse, where the perforin and granzymes penetrate the target cell membrane, ultimately resulting in the death of the target cell via apoptosis. This process, also known as degranulation, is a critical step required for immediate lytic function mediated by responding antigen-specific

$CD8^+$ CTLs [8]. Killing via the granule-independent mechanism involves up-regulation of Fas ligand (FasL) on the surface of the $CD8^+$ CTL following TCR stimulation, which can bind Fas receptors on the surface of the target cells. This initiates several signalling pathways that can lead to apoptosis of the target cell through either a direct caspase cascade or the involvement of the mitochondrial apoptosis machinery [9].

In addition to mediating the killing of target cells, CTLs have been demonstrated to influence the immunological response to a pathogen, via the secretion of various cytokines including tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), as well as chemokines that function to attract and/or activate inflammatory cells to the site of infection [5].

1.3 Cytotoxic T Lymphocytes and HIV

The elimination of acute viral infections such as influenza [10] and RSV [11] and the containment of chronic infections such as CMV [12] and HSV [13] in healthy individuals is mediated by the proliferation and maintenance of virus-specific $CD8^+$ CTLs. Infection with HIV also elicits a strong virus-specific CTL response [14], which initially appears to be effective in recognizing HIV antigens and suppressing viral replication. CTLs play an important role in controlling the progression of HIV disease. This is suggested by the appearance of HIV-specific $CD8^+$ CTLs, which is coincident with a reduction in plasma viremia following primary HIV infection [9-12]. Furthermore, patients demonstrating stronger CTL responses have lower viral loads and slower disease progression [6,8,13,14,15]. Studies performed in macaque monkeys also demonstrate the crucial role $CD8^+$

T cells play in suppressing Simian Immunodeficiency Virus (SIV) replication *in vivo* [16, 17], where $CD8^+$ T cell depletion was associated with a marked increase in SIV plasma viremia and disease progression. Ultimately, however, the initial suppression of viremia is insufficient for complete viral clearance [15], and HIV infection leads to a gradual decline in CTL function. Using HLA-peptide tetrametric staining, Spiegel *et al.* have demonstrated the presence of both HIV- and CMV-specific $CD8^+$ T cells in patients with advanced disease [24]. Thus, it would appear that in the context of advanced HIV infection, virus-specific $CD8^+$ T cells remain present in the circulation but become unresponsive to their cognate antigens.

Impaired $CD8^+$ T cell function also contributes to the susceptibility to opportunistic infections and malignancies. HIV-infected non-progressors have been demonstrated to possess a qualitatively different and presumably superior HIV-specific $CD8^+$ T cell response compared to that of HIV-progressors. It has been suggested that the quality of the $CD8^+$ T cell functional responses rather than the quantity or phenotype of the $CD8^+$ T cells, serves as an immune correlate of HIV disease progression [16]. Many mechanisms including down-regulation of CD3-ζ chain [17, 18], reduced perforin expression [19], reduced IFN-γ expression [20] and maturational arrest of $CD8^+$ effectors T cells [21] have been proposed and evaluated as potential explanations for decreased CTL activity during HIV infection, however, none has been clearly shown to explain the impaired CTL activity. Identifying the reason for this impaired function may

lead to ways of enhancing HIV-specific CTLs and provide significant benefits in the management of HIV-disease.

1.4 The Interleukin-7/Interleukin-7 Receptor System

1.4.1 Expression

Interleukin-7 (IL-7) is a 25 kDa glycoprotein from the hematopoetin cytokine family. It is secreted by stromal epithelial cells of the thymus and bone marrow, as well as a wide array of other cell lineages including keratinocytes, fetal hepatocytes, dendritic cells, platelets and human microvascular endothelial cells including lymph tissue and intestinal epithelium [22]. Circulating levels of IL-7 in healthy individuals range from 0.3-8.4 pg/ml [23, 24], although IL-7 has been shown to bind extensively to components of the extracellular matrix [25, 26], which can localize IL-7 to the microenvironment of production and thereby concentrate IL-7 locally.

IL-7 signals cells via the IL-7 receptor (IL-7R) complex, a unique member of the hematopoeitin receptor family, identified by a Trp - Ser - X- Trp - Ser motif. It consists of two subunits; the IL-7Rα chain, also known as CD127, which binds IL-7, and the common cytokine receptor IL-2Rγ chain, also known as common gamma chain (γc) or CD132, which is shared by the common γ-chain family cytokines IL-2, IL-4, IL-9, IL-15 and IL-21 [27] . The IL-7Rα chain is expressed by various immune cells as well as intestinal epithelial cells and keratinocytes [15]. Expression of the CD127 regulates the effects of IL-7 on T cells. In contrast to IL-7, expression of the gene encoding CD127 increases or decreases during B and T cell development. Constitutively expressed on naïve T cells, the expression of

CD127 by mature T cells is influenced by antigens and cytokines and is down-regulated upon cell activation [28]. DNA microarray analysis has demonstrated that CD127 expression decreases during the transition of naïve to effector T cells following antigen stimulation [29, 30]. CD127 is re-expressed on a small number of T cells after the expansion phase, suggesting that CD127 expression plays a role in the transition from effector to memory T cells [29]. The human IL-7 receptor gene has been located to chromosome 5 band 13 and it encodes a 439 amino acid (aa) transmembrane protein composed of a 219 aa extracellular domain, a 25 aa transmembrane domain and a 195 aa cytoplasmic tail [31]. The murine and human IL-7R cDNAs have both been cloned, and a secreted form of the receptor has also been characterized.

1.4.2 Signalling

Signal transduction pathways associated with IL-7R signalling are not fully defined. IL-7 signalling is initiated when IL-7 binds CD127, triggering hetero-dimerization with the γc and bringing together the tyrosine kinases janus kinase 1 (JAK-1) and JAK-3. The γc-associated JAK-3 is then able to phosphorylate the CD127 and associated JAK-1, creating a docking site for several signalling molecules with Src homology 2 (SH2) domains. Numerous reports have demonstrated that signals transduced by IL-7 initiate from subdomains within the IL-7Rα-chain cytoplasmic tail. These include an acidic amino acid domain, a serine-rich domain and a tyrosine-rich domain containing three tyrosine residues (Y401, Y449 and Y456) [32] as well as a domain known as Box1 that is the binding site for JAK-1 [33]. In studies with T cell lines expressing mutant IL-7R

chimeric receptors, Jianq *et al.* demonstrated that signals originating from Box1 and Y449 are essential to IL-7-mediated T cell survival and proliferation [34]. IL-7/IL-7R interactions allow signal transduction through various signalling pathways including the JAK/STAT (signal transducers and activators of transcription) pathway, the phosphatidylinositol 3-kinase (PI3K) pathway, the mitogen-activated protein kinase (MAPK) pathway, as well as activation of various Src family kinases.

A key signalling molecule that is phosphorylated following recruitment to the CD127 is STAT5. Upon binding to Y449, STAT5a and STAT5b are phosphorylated by JAK1 and JAK3, dimerize and translocate into the nucleus. Once in the nucleus, STAT5 induces transcription of several genes affecting cell survival and proliferation. IL-7R signalling maintains cell survival by promoting a positive balance of the B cell lymphoma 2 (Bcl-2)-family members. It has been demonstrated to increase expression of the survival proteins Bcl-2 and destabilize the cell death proteins BAX (Bcl-2-associated X protein) and BAD (Bcl-2-antagonist of cell death) [35].

IL-7-induced PI3K activation has also been observed and is mediated by JAK3 tyrosine phosphorylation [36]. Once activated, PI3K translocates to the plasma membrane where it phosphorylates phosphatidylinositol bisphosphate 2 (PIP2). Conversion of PIP2 into PIP3, recruits AKT, the major effector of PI3K signalling. AKT is a serine/ threonine kinase with nearly 900 cellular targets [37]. Signalling through the IL-7 receptor also appears to lead to phosphorylation of Pyk2, a member of the focal adhesion kinase family as well as src family kinases

[38]. Details of how these signalling events regulate gene expression, protein production and changes in cellular function remain to be determined.

1.4.3 Regulation

The regulation of surface CD127 expression remains under investigation. CD127 is expressed on the surface of lymphoid cells at various stages of development and is controlled by a promoter region within 197 base pairs upstream of the ATG translation start site on the CD127 gene. At the transcriptional level, regulation of CD127 has been demonstrated to be positively controlled by the PU.1 promoter element in lymphoid cells [39] and in mature T cells by a GA-binding protein [40].

Several types of stimuli are capable of inducing CD127 expression. The glucocorticoid, dexamethasone, has been demonstrated to up-regulate CD127 expression in both naïve and memory $CD4^+$ and $CD8^+$ T cells. In these experiments, CD127 expression was up-regulated at the mRNA and protein levels and was associated with functional changes such as an IL-7 driven increase in surface expression of the IL-2Rα chain (CD25), and enhancement of IL-7 mediated survival signals, preventing cell death *in vitro* [41]. Various stimuli have also been shown to decrease CD127 expression. Surface expression of CD127 is transiently down-regulated during antigen-driven proliferation and has been associated with a loss of Bcl-2 expression [42]. Recent microarray data also suggest that IL-2 negatively regulates expression of CD127 mRNA and protein via a PI3K / Akt-dependant mechanism [43]. In addition, exogenous IL-7 has also been shown to down-regulate CD127 in human T cell and animal models [44-46].

Regulation of IL-7 production has yet to be fully characterized. IL-7 production occurs at a fixed constitutive rate [47] and it has been suggested that serum levels of IL-7 are mainly determined by its consumption rather than its production [35]. The amount of IL-7 is thought to be sufficient to maintain an individual's lymphoid pool; any excess T cells would not survive. Following depletion of the lymphoid pool, new T cells would encounter abundant IL-7 and proliferate until a balance is re-established. This current view of homeostatic control by IL-7 is a unique mechanism and is distinct from other cytokines.

1.5 Interleukin-7: a Cytokine With Central and Peripheral Activity

Originally characterized as a pre-B cell growth factor [48], IL-7 has recently been identified as a cytokine responsible for controlling T lymphopoiesis and peripheral T cell expansion. IL-7 is a cytokine with both central and peripheral activity playing a major role at various stages of T cell development.

1.5.1 Role of IL-7/IL-7R in T cell Development

1.5.1.1 IL-7/IL-7R and Lymphopoiesis

IL-7 is a non-redundant cytokine for both T cell and B cell development. Early T and B lymphocyte production have been demonstrated to be severely impaired in mice deficient in IL-7 or CD127 as well as mice treated with neutralizing antibodies to IL-7 [49]. In humans, IL-7 is critical for the generation of the T cell lineage. As demonstrated, a mutation in the gene coding for the γc chain is sufficient to reduce T cell counts and cause X-linked severe combined immunodeficiency (SCID) [50]. A point mutation in the CD127 gene causing a

partial deficiency in CD127 as well as mutations in JAK3 have also been shown to be sufficient to abrogate T cell development and cause SCID [51]. These findings suggest that IL-7R signalling plays an important role in T cell development and the T cell defects observed in these various forms of SCID may be attributed to defects in IL-7R signalling.

1.5.1.2 IL-7/IL-7R and Thymopoiesis

Significant amounts of IL-7 are produced within the thymus, where it acts as a growth and maintenance factor for immature and mature thymocytes. IL-7 production is crucial for intrathymic maturation, proliferation of immunocompetent T cells and has a critical role in the expansion of the early triple negative (TN) $CD3^-CD4^-CD8^-$ precursors [52]. It has been suggested that *in vivo*, IL-7 promotes survival of thymocytes through the up-regulation of Bcl-2 [53]. The IL-7 receptor also plays an important role in the maturation of thymocytes at various stages of development within the thymus [54]. Down-regulation of IL-7 receptor expression can be seen at the double positive (DP) $CD3^{low}CD4^+CD8^+$ stage whereas up-regulation of the receptor occurs as it enters the single positive (SP) $CD4^+$ or $CD8^+$ stage in normal maturation. In CD127 knockout mice, a block in thymocyte differentiation occurs predominantly at the TN stage, along with impairment of expansion of DP cells [54]. Therefore, further maturation of DP cells to the SP $CD4^+$ or $CD8^+$ involves the up-regulation of CD127. IL-7 also serves as a cofactor for V(D)J rearrangement of the TCR β gene [55].

1.5.2 Role of IL-7/IL-7R in the Regulation of Peripheral T Cell Homeostasis

IL-7 plays a number of roles in T cell homeostasis. Through various pathways, IL-7 and IL-7 receptor signalling are involved in successful thymopoiesis, the survival of naïve T cells, the proliferation of peripheral naïve and memory T cells, as well as in the genesis of memory T cells.

1.5.2.1 IL-7/IL-7R in Naïve T Cell Homeostasis

The association of IL-7 with the maintenance of the naïve T cell pool was first demonstrated by Tan *et al.*, where the transfer of fluorescently labelled naïve $CD4^+$ and $CD8^+$ T cells into IL-7 knockout mice results in rapid loss of these transferred naïve cells [56]. IL-7 and IL-7 receptor signalling have anti-apoptotic effects on T cells by upregulating Bcl-2 [28] and it is through the provision of these survival signals, that IL-7 plays an important role in the post-thymic maintenance of naïve T cells.

1.5.2.2 IL-7/IL-7R and T Cell Proliferation

Numerous murine as well as human models have demonstrated the involvement of IL-7 in controlling homeostasis via homeostatic driven proliferation of T cells in the periphery. IL-7 stimulates the homeostatic expansion and survival of both naïve $CD4^+$ and $CD8^+$ T cells [28, 57, 58]. IL-7 can also support the homeostatic expansion and survival of $CD4^+$ and $CD8^+$ memory T cells [58, 59]. Importantly, IL-7 has been found to expand human T cells while maintaining $CD45RA^+$ naïve phenotype, without switching the phenotype to $CD45R0^+$ [60, 61].

1.5.2.3 IL-7/IL-7R and Memory T Cell Genesis

The generation of memory T cells is linear and progressive. Antigenic stimulation causes naïve T cells to undergo an expansion phase, resulting in the generation of Th (T helper) 1 and Th2 cells. This expansion phase is followed by a death phase, when 90 to 95% of the effector T cells die. The surviving 5 to 10% of the effector T cells further differentiate into an effector memory T cell population (TEM), which continue to differentiate into central memory T cells (TCM) in the absence of antigen, and acquire the ability to persist in the periphery via homeostatic proliferation [62]. IL-7 is not required for the initiation of the antigenic response, however, following clonal expansion of antigen-specific T cells, IL-7 is essential for the generation of memory T cells (TEM and TCM), both in the $CD4^+$ and $CD8^+$ T cell compartments [29, 63]. T cells that survive this expansion phase retain CD127 expression, suggesting that IL-7 receptor signalling plays a role in the genesis and maintenance of memory T cells.

1.5.3 Role of IL-7/IL-7R in Immune Responses

1.5.3.1 IL-7/IL-7R and Th1/Th2 Responses

Antigenic stimulation of naïve $CD4^+$ T cells leads to the development Th cells. T helper cells secrete a variety of cytokines, which determine the profile of the immune response. Th1 cells produce IFN-γ and control the cell-mediated immune response, while Th2 cells produce IL-4 and control the humoral immunity. IL-7 has been demonstrated to enhance IFN-γ production several ways: 1) by synergizing with IL-12 to increase IFN-γ production [64] and 2)

increase IFN-γ production in co-stimulation with αCD3/αCD28 [65]. It has been suggested that IL-7 is an intermediate cytokine in the Th1 immune response [66].

1.5.3.2 IL-7/IL-7R in inducing CTL Responses and NK Activity

Several cytokines have been demonstrated to control different steps in the generation of an effective CTL response, including IL-7. In human influenza A virus-stimulated peripheral blood mononuclear cells (PBMCs), IL-7 enhanced cytotoxicity of CTL against virus-infected autologous target cells [67]. In mixed lymphocyte culture (MLC), IL-7 has been found to increase the cytolytic activity as well as induce the generation of CTLs, suggesting that IL-7 has potent regulatory effects on human cytolytic cell populations [68]. IL-7 has also been shown to increase CTL activity by increasing total serine esterase activity [67], as well as pore-forming protein [64, 69]. Additionally, recent publications have described IL-7 as being capable of generating lymphokine-activated killer cell (LAK) activity in PBMCs [68, 70, 71], where $CD56^+$ natural killer (NK) cells were the predominant LAK cells in this response [70].

1.6 The Impact of IL-7/IL-7R Signalling on $CD8^+$ T cells and CTL Activity

IL-7 and IL-7 receptor signalling are vital for the survival and function of $CD8^+$ T cells. In addition to being crucial to T cell precursors in the bone marrow, IL-7 and IL-7 receptor signalling are essential to mature $CD8^+$ T cells in the periphery. IL-7 sustains lymphopoiesis and the production of T cell precursors, without which we would not have any $CD8^+$ T cells. IL-7 and IL-7 receptor signalling are key in the expansion of the early triple negative (TN) $CD3^-CD4^-CD8^-$ precursors, intratrathymic proliferation as well as maturation of T cells [52],

hence it is required for precursor T cells to become $CD8^+$ T cells that exit the thymus.

Once out of the thymus, IL-7 and IL-7 receptor signalling are necessary for the maintenance/survival of naïve $CD8^+$ T cells via up-regulation of Bcl-2, and homeostatic expansion of naïve and memory $CD8^+$ T cells via proliferation. During an infection, the transition from TEM $CD8^+$ T cells to TCM $CD8^+$ T cells is also dependent on IL-7 and IL-7R signalling. In well characterized murine models of developing immune responses to viral infection employing LCMV infection, Kaech *et al.* demonstrated the dependence of memory $CD8^+$ T cell genesis on IL-7 and that IL-7Rα expression on $CD8^+$ T cells distinguishes those TEM cells that successfully develop into TCM cells [29]. In 1991, Smyth *et al.* demonstrated that IL-7 augments the cytotoxic potential exclusively in $CD8^+$ T cells, by inducing pore forming protein mRNA in $CD8^+$ T cells in a dose dependent manner [69]. In 1990, Alderson *et al.* demonstrated that in MLC, IL-7 augments both the cytolytic activity of T cells as well as the number or generation of anti-viral CTLs. Cell surface phenotypic analysis of IL-7-generated CTL effector cells revealed that $CD8^+$ T cells were responsible for the vast majority of lytic activity. Since CTLs play an important role in limiting viral replication and eradicating virus-infected cells in HIV infections, being able to augment their cytolytic functions or their numbers would be beneficial. Once again, this illustrates the importance of IL-7 to $CD8^+$ T cells.

1.7 IL-7/IL-7R system in Human diseases

Disregulation within the IL-7/IL-7R system has been identified in several human diseases. Increased IL-7 levels have been detected in patients with septic shock [72], graph versus host disease (GVHD) [73] as well as juvenile rheumatoid arthritis [74]. In hepatitis C virus (HCV) infections, CD127 expression is down-regulated on total $CD4^+$ and $CD8^+$ T cells, as compared to healthy controls and it has been suggested that CD127 is a useful marker of functional $CD4^+$ and $CD8^+$ T cells as its expression correlates with virologic outcome of acute HCV [75]. Down-regulated CD127 expression as well as impaired responsiveness to IL-7 has also been observed in peripheral blood mononuclear cells (PBMCs) from cancer patients [76]. Several groups have also identified associations between genetic polymorphisms in the human CD127 and human disease including multiple sclerosis (MS) [77, 78] and inhalation allergy [79]. These types of polymorphisms may influence ligand binding and/or the interaction between the CD127 and the common γ-chain and thereby influence the biological activities of IL-7.

1.7.1 IL-7 in HIV Disease

HIV-infected patients have increased levels of IL-7. Napolitano *et al.* proposed that IL-7 production increases as a part of the homeostatic response to T-cell depletion in HIV disease and demonstrated that increased levels of IL-7 are strongly associated with $CD4^+$ T lymphopenia and that IL-7 production is greatly increased in lymphocyte-depleted tissues [80]. It was theorized that because the IL-7 receptor is expressed on mature T cells, circulating levels of IL-

7 may be higher when $CD4^+$ T cells counts are low, simply because fewer receptors are available. However, due to elevated numbers of $CD8^+$ T cells in HIV disease, it was speculated that it is more likely that there is a down-regulation in CD127 on T cells, resulting in less bound and therefore more circulating IL-7. Since the number and proportion of B- and T-lymphocytes in both naïve and memory compartments must be sustained to maintain immunocompetance, altered IL-7 levels may reflect a mechanism by which the body responds to disturbances in lymphocyte compartments. Given these observations, it is possible that IL-7 will enhance the HIV-specific CTL response in infected individuals. Despite the increase in IL-7 levels during HIV infection, CTL activity becomes progressively impaired with advanced disease suggesting an inability to respond to IL-7.

In order to determine responsiveness to IL-7 in HIV disease, the ability of IL-7 to support the activation and growth of *in vitro* antigen-specific CTL precursors present in PBMCs from HIV-infected patients was studied by Ferrari *et al.* The inclusion of IL-7 in a vaccinia/HIV-1 vector-based stimulation strategy greatly augmented overall CTL reactivity. In four out of six patients, HIV-specific lytic activity was significantly higher in cultures stimulated with antigen plus IL-7 than those with antigen alone. Cytolytic activity was principally mediated by $CD8^+$ effector T cells. $CD3^+/CD8^+$ T cell expansion was also increased by 2·7-fold in the presence of IL-7 and cytofluorimetric analysis revealed that IL-7 preferentially expanded $CD8^+$ memory T cells ($CD45RO^+$) and $CD8^+$ lymphocytes expressing activation molecules [81]. These data taken together indicate a partial

responsiveness to IL-7 in HIV infection but do not address how this compares to the effect of IL-7 on CTL effector function in HIV negative individuals.

1.7.2 CD127 Expression in HIV Disease

In 1994, Carini *et al.* investigated IL-7 responsiveness of $CD8^+$ T cells from ten HIV^+ patients, five of which were positive and five negative for HIV-specific CTL activity [82]. Addition of IL-7 to PBMCs from these patients increased the cytotoxic response only in the five individuals who originally demonstrated CTL activity. When examined by flow cytometry, the PBMCs from the remaining five lacking an HIV-specific CTL response were found to have a marked decrease in the percentage of $CD8^+$ T cells expressing CD127, the receptor for IL-7. Therefore, it is possible that the decline in CTL activity in HIV infection is due to a decrease in the expression of the IL-7 receptor.

Our demonstration that significantly fewer $CD8^+$ T cells from HIV infected patients express CD127 as compared to healthy individuals [83], supports the hypothesis that the decline of CTL activity seen in HIV infection is a result of impaired CD127 expression. When patients on effective therapy with sustained viral suppression were examined, a greater proportion of $CD8^+CD127^+$ T cells was noted as compared to untreated individuals, suggesting that ongoing HIV replication either directly or indirectly contributes to CD127 down-regulation and that this plays a role in HIV immunopathogenesis. Decreased CD127 expression in HIV disease has also been recently demonstrated by several other groups [84, 85]. In addition to $CD8^+$ T cells, CD127 expression on $CD4^+$ T lymphocytes decreases with HIV

disease progression and has been demonstrated to inversely correlate with immune activation [86]

1.8 IL-7 Immunotherapy for HIV

The IL-7/IL-7Rα system plays an essential as well as a non-redundant role in human T cell development, and acts as a central T cell regulator affecting the survival and homeostasis of naïve and memory $CD4^+$ and $CD8^+$ T cell subsets. The capability of IL-7/IL-7R signalling to induce proliferation of immature thymocytes may be key in increasing thymic output and enhance immune reconstitution of naïve T cells following HIV-induced lymphopenia. IL-7/IL-7R signalling's role in TCR rearrangement during thymopoiesis could lead to increasing the T cell repertoire during HIV disease, and their role in inducing Bcl-2 expression may help in the survival of thymocytes. In the periphery, the proliferative effect IL-7/IL-7R signalling have on mature circulating T cells could increase peripheral T cell expansion. Similarly to aiding in the survival of thymocytes, IL-7/IL-7R signalling may help peripheral T cell survival via the induction of Bcl-2. Furthermore, IL-7's role in inducing immune responses may be essential when considering IL-7 as an immunotherapeutic. IL-7 has been demonstrated to increase CD8-specific cytotoxicity against viral antigens, increase IFN-γ secretion, and promote a Th1 response, all of which would be beneficial in minimizing HIV-disease progression.

Clinical development of IL-7 is currently underway. Several studies in mice [87, 88] and primates [46, 89] have demonstrated that IL-7 therapy exerts marked effects on T cell immune reconstitution and have implicated IL-7 as a primary

modulator of peripheral T cell homeostasis with potent immunorestorative properties. In 2008, Sportes *et al.* were the first to demonstrate that IL-7 administration in humans can safely induce polyclonal T cell expansion *in vivo*, which resulted in a dramatic increases in T cell number as well as the expanded of naïve $CD4^+$ and $CD8^+$ subsets. They suggested that IL-7 appears to be an effective T cell growth factor with " immune rejuvenating " properties, augmenting immune reactivity in hosts with impaired immunity caused by physiological (age), iatrogenic (chemotherapy/transplantation), or pathological (HIV) lymphodepletion [90].

Before the use of IL-7 in clinical trials in HIV-infected individuals, a careful examination of possible adverse side effects is required. One concern regarding its *in vivo* effects is the possibility that IL-7 may increase infectivity of HIV by increasing HIV replication and upregulating expression of chemokine receptors, hence promoting viral pathogenicity. *In vitro*, IL-7 has been demonstrated to increase HIV replication in CD8-depleted PBMCs from HIV-infected individuals [91, 92], and to induce expression of latent HIV-1 in chronically infected cells [93]. In $CD4^+CD8^-CD3^+$ thymocytes, IL-7 has been demonstrated to sustain expression of the p75 TNF receptor, allowing TNF-α to induce high levels of HIV-1 replication exclusively in these mature $CD4^+CD8^-CD3^+$ thymocytes [94]. In terms of promoting viral pathogenicity, IL-7 has been reported to upregulate CXCR4 chemokine receptor expression in thymocytes [95] and PBMCs [96]. Higher levels of circulating IL-7 have been detected in patients harbouring CXCR4 HIV-1 variants [97]. However, of partial reassurance is the discovery that

IL-7 stimulates T cell renewal without increasing viral replication in SIV-infected macaques in the absence of antiretroviral therapy [98], suggesting that IL-7 therapy may not have a negative impact in terms of furthering HIV replication and disease.

Another limiting factor in the use of IL-7 as a therapeutic is the suggestion that IL-7 therapy has the potential to induce or aggravate autoimmunity in pre-disposed individuals [99]. Furthermore, the implication of the IL-7/IL-7R system in generating neoplastic events in lymphocytes is of great concern to any potential use of IL-7 as a therapeutic. Preliminary data from administration of IL-7 to non-human primates with HIV and to patients with metastatic cancer did not result in excess cases of lymphoma or other malignancies [100-102] [103]. However, this needs to remain an area of ongoing investigation, and Phase I/II human clinical trials which are designed to look at short-term safety and tolerability may not be appropriate for monitoring the development of lymphomas, which may take years to develop.

Despite these potential unwanted effects, IL-7 is an immunotherapeutic candidate that brings hope to HIV patients. IL-7 immunotherapy could help restore CD4 counts and promote a specific anti-HIV response in HIV infected individuals.

1.9 Rational

The reason for, or mechanism of IL-7 receptor down-regulation is unknown although progressive cytokine dysregulation along with disrupted lymph node [58] and thymic architecture [59], during HIV infection could affect IL-7 receptor expression on $CD8^+$ T cells. If this were the case and the IL-7 receptor was either down regulated on pre-existing $CD8^+$ T cells or not expressed during $CD8^+$ T cell maturation, impaired proliferation and activation would be expected. With disease progression, the $CD8^+$ T cell population would then be driven towards an anergic state. As an increase of IL-7 occurs in the face of declining CTL activity, providing additional IL-7 is unlikely to enhance HIV specific CTL activity. On the other hand, enhancing IL-7 signalling by increasing the expression of, or enhancing the function of IL-7 receptors may result in improved $CD8^+$ T cell function and enhanced control of viral replication. To this end, identifying the abnormalities of CD127 expression and function during HIV infection, determining the mechanisms thereof and describing the potential restoration with effective antiretroviral therapy may provide insights into the development of much needed immune-based therapies for patients with HIV infection.

1.10 Hypothesis

Altered IL-7 receptor (CD127) expression and function on $CD8^+$ T cells in HIV infection contributes to impaired cell mediated immunity.

1.11 Specific Aims

1) To determine which host or viral factors may be responsible for the down-regulation of CD127, on $CD8^+$ T cells in HIV infection.

2) To determine the mechanism(s) by which these host and/or viral factors decrease CD127 expression on $CD8^+$ T cells:

3) To determine if *in vitro* infection with HIV down-regulates CD127 expression on $CD8^+$ T cells

4) To determine if the functional capacity of CD127 is altered in HIV infection and if this is restored with suppression of viral replication.

2 MATERIALS AND METHODS

2.1 Viral Stocks

The T-tropic HIV-1_{IIIB} and the M-tropic HIV-1_{Ba-L} were obtained from the National Institute of Health AIDS Reagent Program (Rockville, MD). The dual-tropic HIV-1 clinical isolate cs204 was obtained from Dr. Diaz-Mitoma (University of Ottawa).

2.1.1 Preparation and Expansion of HIV-1 Virus Stocks

HIV-1_{IIIB} and HIV-1_{cs204} were expanded in Jurkat cells. Jurkat cells (10 x 10^6) were resuspended and cultured in 1 ml of supernatants of appropriate virus or 1 ml of RPMI (for Mock HIV-1) for 4 hours at 37°C and 5% CO_2, after which time, cells were resuspended in RPMI + 10% FBS in tissue culture flasks at 0.5 x 10^6 cells/ml. Every 3-4 day of culture, 10 ml of fresh RPMI $^+$ 10% FBS was added to the THP-1 cells. HIV-1_{Ba-L} was expanded in 3-day phytohaemagglutinin (PHA) and IL-2 stimulated PBMCs. PBMCs (30 x 10^6) were cultured at 1 x 10^6 cells/ml in RPMI +10% FBS + PHA (5 μg/ml) + IL-2 (100 U/ml). On day 3 of stimulation, 10 x 10^6 PBMCs were resuspended and cultured in 1 ml of virus supernatant or 1 ml of RPMI (for Mock HIV-1) for 4 hours at 37°C and 5% CO_2, after which time, cells were resuspended in RPMI + 10% FBS + PHA (5 μg/ml) + IL-2 (100 U/ml) in tissue culture flasks at 1x 10^6 cells/ml. Every 3-4 day of culture, 10 ml of fresh RPMI + 10% FBS + PHA (5 ug/ml) + IL-2 (100 U/ml) was added to PBMCs. On days 0, 7 and 14 of culture, 1 ml aliquots of culture supernatants were harvested from HIV and mock infected cells, and stored at -80°C. For HIV-1 virions

defective in reverse-transcriptase activity,1 ml aliquots of culture supernatants were harvested from 8e5 cells, and stored at -80°C. HIV-1 replication was verified by assaying the culture supernatants for HIV-1 p24 protein production by enzyme-linked immunosorbent assay (ELISA). Cell-free virus containing supernatants were harvested and stored at -80°C.

2.1.2 Determination of HIV-1 p24 in Viral Stocks

Concentration of p24 in viral stocks was determined using a HIV-1 p24 Capture ELISA (ImmunoDiagnostics, Woburn MA, USA) in accordance with manufacturer's instructions. In Brief, 10 µl of 10% Triton X/phosphate buffered saline (PBS) was added to 200 µl of viral stock (to achieve a final concentration of 1% Triton X) and incubated for 60 minutes at 37°C to kill any viral particles. Viral stock samples as well as appropriate diluted standards (25-250 000 pg/ml) were aliquoted into one well of the ELISA plate and incubated for an h at room temperature followed by horseradish peroxidase (HRP)-conjugated p24 detector reagent, 3, 3', 5, 5'-tetramethylbenzidine (TMB) and stop reagent. Plates were read at 450 nm on a SpectraMax 190 microplate reader (MDS Analytical Technologies). Calculation of protein content in each sample against the standard curve was performed using the SoftMax Pro software.

2.1.3 $TCID_{50}$ Determination of Viral Stocks

The 50% tissue-culture infectious dose ($TCID_{50}$) of each viral stock was determined in PBMCs. In brief, 50 x 10^6 PMBCs (1 x 10^6 cells/ml) were cultured in RPMI + 10% FBS + PHA (5 µg/ml) + IL-2 (100 U/ml) at 37°C and 5% CO_2. On

day 3 of PBMC stimulation, a viral stock was diluted 1:12 in RPMI + 10% FBS + PHA (5 μg/ml) + IL-2 (100 U/ml) and 200 μl was aliquoted in triplicate into one row of a 96 well plate. To the next 6 rows, in triplicate, 150 μl of RPMI + 10% FBS + PHA (5 ug/ml) + IL-2 (100 U/ml) was added. Next, 50 μl of diluted virus from the first row was transferred to the second row and so on, producing a series of 4-fold dilutions of virus across the 6 rows. Finally, to every well in the plate, 50 μl (4 x 10^6 cells/ml) of stimulated PBMCs were added, and the plate was incubated at 37°C and 5% CO_2. On day 4 of culture, 150 μl of medium was discarded from each well and replaced with fresh medium (RPMI + 10% FBS + PHA (5 μg/ml) + IL-2 (100 U/ml)). On day 7 of culture, supernatants from each well were harvested, centrifuged and assayed for HIV-1 p24 protein expression by ELISA. Wells in which the p24 content exceeded 50 ng/ml were scored as positive, and the $TCID_{50}$ for the viral stock was calculated using the Spearman-Karber Method as follows:

$$M = xk + d[0.5 - (1/n)(\Sigma r)]$$

Where,

xk = the dose of highest dilution

r = the number of negative wells

d = the spacing between dilution

n = the number of wells in each dilution

Therefore, the 50% endpoint equals 4^{-M}

And to convert to the 50% titer (10^x), x = M•log4

Finally, the $TCID_{50}$/ml is determined by multiplying by 5, to correct for the original dilution (i.e. 1000 ul / 200 ul)

2.2 Preparation of Cells

2.2.1 Cell Lines

The WI, the Jurkat and 8e5 cell lines were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). All cell lines were maintained in T-75 tissue culture flasks in RPMI-1640 medium (Invitrogen, Carlsbad CA, USA) supplemented with 10% heat-inactivated fetal calf serum (FBS) (Cansera, Etobicoke ON, Canada), 100 IU/ml each of penicillin and streptomycin (Sigma-Aldrich, Oakville ON, Canada), and incubated at 37°C and 5% CO_2. The THP-1 as well as 8e5 cell lines were maintained in suspension and cells were replaced at a density of 0.5 x 10^6 cells/ml every 3-4 days. The WI cell line was maintained in a single monolayer and cells were trypsinized and replaced at a density of 0.3 x 10^6 cells/ml every 2-3 days.

2.2.2 Peripheral Blood Mononuclear Cells (PBMCs)

All research conducted using blood from human subjects was approved by the Ottawa Health Research Ethics Board. Blood from HIV-seronegative donors was collected into heparin-containing tubes and PBMCs were isolated by Ficoll-Paque™ PLUS (Pharmacia Fine Chemicals, Oiscataway NJ, USA) gradient separation following manufacturer's instructions. Blood was overlaid on Ficoll-Paque™ PLUS in 50 ml conical centrifuge tubes at a ratio of 2:1 and centrifuged

at 1600 rpm for 30 minutes with no brake. Next, the buffy coat layer was collected and washed twice in 5-fold excess PBS. PBMCs were counted by trypan blue and cultured ($1x10^6$ cells/ml) in complete RPMI Medium (Invitrogen) supplemented with 10% FBS (Cansera), 100 IU/ml each of penicillin and streptomycin (Sigma-Aldrich).

2.2.3 Purified $CD8^+$ T Cells

The $CD8^+$ T cells were isolated from PBMCs using a MACS $CD8^+$ T Cell Isolation Kit (Miltenyi Biotec, Auburn CA, USA) in accordance with manufacturer's instructions along with the autoMACS cell sorter's "posel" protocol to remove labeled cells, achieving a purity of > 98-99 % as verified by flow cytometry. PBMCs were resuspended in 80 µl of MACS buffer (PBS $^+$ 0.5% bovine serum albumine (BSA) (Sigma-Aldrich) $^+$ 2mM ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich)) and 20 µl of MACS CD8 MicroBeads per 10^7 total cells, and incubated for 15 minutes at 4°C. Cells were then washed in 10-fold excess MACS buffer, and magnetically separated. Purified $CD8^+$ T cells were cultured ($1x10^6$ cells/ml) in complete RPMI Medium (Invitrogen) supplemented with 20% FBS (Cansera), 100 IU/ml each of penicillin and streptomycin (Sigma-Aldrich).

2.3 Cell Culture Conditions for Evaluating the Role of Viral/Host Factors on Surface CD127 Expression

The PBMCs and purified $CD8^+$ T cells were cultured with increasing concentrations of HIV-1gp120 [1, 5 and 10 µg/ml], HIV-1tat [0, 0.1, 1 and 10 µg/ml] or HIV-1nef (0, 1 and 10 µg/ml) (NIH AIDS Research and Reference

Reagent Program, Division of AIDS, NIAID, NIH, from ImmunoDiagnostics, Woburn, MA); recombinant human IL-7 [10, 100, 1000 and 10 000 pg/ml] (R & D, Minneapolis, MN); IL-1-β,IL-4, IL-6, IL-10, IL-13 [1, 10 and 100 ng/ml] (BD Biosciences Pharingen, San Diego, CA); TGF-β [1, 10 and 100 ng/ml]; IFN-α [1, 10, 100 ng/ml]; or TNF-α [1, 10 and 100 ng/ml] (Sigma-Aldrich). Cells were cultured in 96-well flat bottom plates at 37°C and 5% CO_2. Following incubations of 0, 24, 48, 72, and 96 hours, CD127, CD45RA, and CD45R0 expression on $CD8^+$ T cells was analysed by flow cytometry. Cell culture conditions for evaluating the role on in vitro HIV-1 on surface CD127 expression

2.4 Cell Culture Conditions for Evaluating the Role of *In Vitro* HIV-1 Infection on Surface CD127 Expression

2.4.1 In vitro HIV-1 Infection of PBMC and Purified $CD8^+$ T Cells

Prior to HIV infection, PBMCs or purified $CD8^+$ T cells were pre-treated with 2 μg/ml of polybrene (Sigma-Aldrich) for 1 hour at 37°C and 5% CO_2 and washed 2 times in 5-fold excess PBS. Cell pellets were infected with HIV-1_{CS204}, HIV-1_{IIIB}, HIV-1_{Bal} or mock infected for 3-4 hour at 37°C and 5% CO_2 and washed in PBS. Infection dose used was a $TCID_{50}$ of 300. For the mock condition, cell pellets were incubated with an equal volume of supernatants to that of the HIV strain used. PBMCs and purified $CD8^+$ T cells were also treated in parallel with supernatants from 8e5 cells containing HIV-1 virions defective in reverse-transcriptase activity (10 ng HIV-1 p24/ml).

2.4.2 Culturing Conditions

2.4.2.1 Examining Direct Role of HIV-1 on CD127 Surface Expression

Following *in vitro* infection, PBMCs or purified $CD8^+$ T cells were resuspended at 1 x 10^6 cells/ml in complete RPMI supplemented 10% FBS (Cansera) (for PBMCs) or 20% FBS (for $CD8^+$ T cells), 100 IU/ml each of penicillin and streptomycin (Sigma-Aldrich). PBMCs or purified $CD8^+$ T cells were culture in 96-well plates at 1 x 10^5 cells/well at 37°C and 5% CO_2 for 24, 48, 72 and 96 hours, at which time cells were stained and subjected to flow cytometry.

2.4.2.2 Examining the Role of Soluble Factors in in vitro *HIV-1-Mediated Decrease in Surface CD127*

Uninfected purified $CD8^+$ T cell were co-cultured with infected allogeneic PBMCs. 5 x 10^5 HIV-1, mock or replication incompetent HIV-1 infected PBMCs were aliquoted into a 24 well plate and 1 x 10^5 purified CD8 were cultured in a transwell placed on top of the PBMCs. Cells were cultured at 37°C and 5% CO_2 for 24, 48, 72 and 96 hours, at which time the purified $CD8^+$ T cells were stained for CD127 and CD8 and subjected to flow cytometry (see above). In separate experiments, uninfected purified $CD8^+$ T cells were resuspended and cultured in supernatants of infected allogeneic PBMCs. 1 x 10^6 HIV-1, mock or replication incompetent HIV-1 infected PBMCs were cultured in a 24-well plate at 37°C and 5% CO2. Following 24 h of culture, supernatants were centrifuged to remove any cells. In parallel, allogeneic purified $CD8^+$ T cells were centrifuged and $CD8^+$ T cells were resuspended at 1 x 10^6 cells/ml in the supernatants from the 24 hours infected PBMCs. Following another 24, 48 and 72 hours of culture at 37°C and

5% CO_2, the purified $CD8^+$ T cells were stained for CD127 and CD8 and subjected to flow cytometry.

2.4.3 Neutralization of IL-7

To delineate any potential role of IL-7 in HIV-1-mediated down-regulation of CD127, infected PBMCs were also treated with neutralizing antibody against IL-7. Following HIV-1, mock or replication incompetent HIV-1 infections, PBMCs were resuspended in media as described above and treated with 10 μg/ml of anti-IL-7. Following 24, 48, 72 and 96 hours of culture at 37°C and 5% CO_2, the PBMCs were stained for CD127 and CD8 and subjected to flow cytometry.

2.4.4 IL-7 and IL-4 ELISA

To quantitate IL-4 or IL-7 protein production by HIV-1cs204 infected PBMCs, cryopreserved supernatants were thawed and assayed for IL-4 or IL-7 protein content by commercial ELISA (BioRay). All ELISAs were performed in accordance with manufacturer's instructions. Briefly, 10 μl of 10% Triton X/PBS were added to 200 μl of viral stock and incubated for 60 minutes at 37°C to kill any viral particles. Supernatants or serially diluted IL-4 (200 to 3.13 pg/ml) or IL-7 (1000 to 4.1 pg/ml) protein standards were added to individual wells of the ELISA plates and incubated over night at 4°C, followed by biotinylated anti-human IL-4 or IL-7 detection antibody, HRP-conjugated streptavidin, TMB and stop reagent. After each step, the ELISA plates were washed 4 times with wash buffer. Plates were read at 450 nm on a SpectraMax 190 microplate reader (MDS Analytical

Technologies). Calculation of protein content in each sample against the standard curve was performed using the SoftMax Pro software.

2.5 Statistical Analysis

The Student's *t*-test or paired *t*-test was used for data analysis of as appropriate. Analysis of variance (ANOVA) was used for multiple group comparisons. Dunnett's test was used after ANOVA has rejected the hypothesis of equality of the means of the distributions, to identify groups whose means are significantly different from the mean of the control group. All statistical analysis was performed using Sigma Stat 3.0 software (SPSS, Leesburg, VA) where values of $p \leq 0.05$ were considered statistically significant.

2.6 Flow Cytometric Analysis of Surface CD127

Flow cytometry analyses were performed using a Beckman Coulter ALTRA flow cytometer and the EXPO version 2.0 software package. Samples of 2 X 10^5 cells were incubated in FACS tubes with saturating antibody concentrations for 20 minutes at room temperature in the dark. Monoclonal antibodies used included CD8-PC5 and CD127-PE, for two-color analysis; CD8-PC5, CD127-PE and CD45RA-FITC or CD45RO-FITC, for thee-color analysis; and CD8-PC5, CD127-PE, CD45RA-ECD and CD27-FITC or CD62L-FITC, for four-color analysis. All monoclonal antibodies were purchased from Immunotech (Beckman Coulter, Marseille, France). The impact of a given stimulus on the expression of CD127 on $CD8^+$ T cells was calculated as follows:

$$\left[\frac{\%CD8^{+}\text{ T cells expressing CD127 in the presence of stimulus}}{\%\text{CD8 + T cells expressing CD127 in media alone}}\right]X100$$

To exclude potential cell mortality as an explanation for observed results, purified $CD8^{+}$ T cells incubated with media alone or the stimulus were stained with 7-aminoactinomycin D (7-AAD) or propidium iodide (PI) (Invitrogen) and samples were analyzed by flow cytometry.

2.7 CD127 mRNA Quantification

2.7.1 CD127 mRNA Quantification From Purified $CD8^{+}$ T Cell Cultures

Following 16 and 24 hour incubations with IL-7 (10 000 pg/ml), IL-4 (100 ng/ml) or 24 hours with HIV-1tat (1 µg/ml) in 6-well flat bottom plates, total RNA was isolated from purified $CD8^{+}$ T cells cultures (3 x 10^{6} cells) using the RNeasy® Mini Kit (Qiagen, Valencia CA, USA) according to manufacturer's instructions. In brief, cells were lysed and homogenized with 350 µl of Buffer RLT and a 20-gauge needle. To the homogenized lysates, 350 ul of 70% ethanol was added and the lysates + ethanol were transferred to an RNeasy mini column and centrifuged at 12 000 x g for 15 seconds. The flow-through were discarded and 80 µl of the DNase I incubation mix was added to the column, incubated for 15 minutes at room temperature and washed with 350 µl of Buffer RW1 with centrifugation of 12 000 x g for 15 seconds. The column was washed twice more with 500 ul of buffer RPE with centrifugation at 12 000 x g for 15 seconds. RNA was eluted from the colums with 30 µl RNase-free water and centrifugation of 12 000 x g for 1 minute. Total RNA concentrations and purity was determined by

measuring absorbance at 260 nm and 280 nm wavelengths, using a SpectraMAX 190 spectrophotometer. RNA concentration was calculated as A_{260} x 40 ug/ml and nucleic acid purity was calculated as the ratio of A_{260}/A_{280}. Samples with ratios between 1.8 and 2.0 were considered acceptable and used for PCR.

2.7.2 CD127 mRNA Quantification From PBMC Cultures

6 x 10^6 PBMC were plated into 6-well tissue culture plates and treated with or without IL-7 (10 000 pg/ml) or IL-4 (100 ng/ml). After 16 and 24 hours of culture at 37°C and 5% CO2, $CD8^+$ T cells were isolated from the PBMC cultures using the MACS CD8 T cell Isolation kit as described previously. Total RNA was then extracted from the purified $CD8^+$ T cells.

2.7.3 Determination of CD127 mRNA Expression in $CD8^+$ T Cells by Semi-Quantitative PCR

cDNA synthesis was conducted using the Advantage RT-for-PCR Kit (Clontech) as follows: 0.5 micrograms of total RNA (in 10 µl distilled water) and 1 µl of $Oligo(dT)_{18}$ were heated to 70°C for 2 minutes and added to 4 µl 5X reaction Buffer, 1 µl of dNTP mix (10mM each), 0.5 µl of recombinant RNase inhibitor and 1 µl of MMLV reverse transcriptase. The mixture was incubated at 42°C for 1 h and 94°C for 5 minutes to stop the cDNA synthesis reaction. Complementary DNA was PCR-amplified with gene-specific primers for the CD127 gene (forward 5'-GAAGGTTGGAGAAAAGAGTC-3' and reverse 5'-CAAAATGCTGATGGTTAGTAA-3'), to amplify cDNA encoding membrane-bound CD127. All semi-quantitative PCRs included 2 µl cDNA, 50 µl of *Taq* PCR Master Mix (Qiagen), 2 µl each of forward and reverse primers (0.3 microM) and 44 µl of

distilled water, for a final volume of 100 µl. Cycling conditions were 30 seconds at 95°C, 30 seconds at 52°C and 1 minute at 72°C, for 30 cycles. PCR products were resolved by electrophoresis on 1% agarose gel, stained with ethidium bromide, and visualized using a UV transluminator.

2.7.4 Determination of CD127 mRNA Expression in $CD8^+$ T Cells by Real-Time PCR

First strand cDNA synthesis was conducted using the SuperScript II Reverse Transcriptase Kit (Invitrogen) as follows: 0.5 micrograms of total RNA (in 10 µl sterile water), 1 µl of $Oligo(dT)_{12\text{-}18}$ (500 µg/ml) and 1 µl of dNTP Mix (10 mM each) were heated to 65°C for 5 minutes and added to 4 µl 5X First-Strand Buffer, 2 µl of 0.1 M DTT and 1µl (200 units) SuperScript II RT. The mixture was incubated at 42°C for 50 minutes and 70°C for 15 minutes. In addition to the use of DNase in sample preparation, the possibility of genomic DNA contamination was addressed by routinely conducting PCR analysis of some RNA samples incubated with reverse transcription reaction mixtures lacking the reverse transcriptase.

Complementary DNA was PCR-amplified with gene-specific primers for the CD127 gene (see previous section). All quantitative PCRs included: 25 µl of QuantiTect SYBR Green PCR Master Mix (Qiagen, Mississauga, ON), 3 µl each of forward and reverse primers (0.3 microM), 10 µl of a 1:100 dilution of cDNA, 1 µl $MgCl_2$ (50 mM) and 8 µl of Rnase-free H_2O for a final 50 µl reaction. Reactions were performed in a BIO-RAD iCycler iQ (BioRad) with a 15 min activation of DNA polymerase at 95°C, followed by 50 cycles of denaturation at 95°C for 30

seconds, annealing at 60.3°C for 20 seconds, extension at 72°C for 30 seconds and measurement of sample fluorescence at 72°C for 5 seconds for quantification. Melting curve analysis confirmed the amplification of a single product with minimal primer-dimer formation. The house-keeping gene ribosomal protein S18 was amplified as above without adding $MgCl_2$. S18 primers (forward 5'-CTGCCATTAAGGGTGTGG-3' and reverse 3'-TCCATCCTTTACATCCTTCTG-5') were designed using Beacon software 3.0. The CD127-specific PCR products were sequenced and homology to the known CD127 sequence was confirmed (data not shown), thereby confirming both primer-specificity and suitability of the PCR product for use in generating standard curves for real-time PCR. To quantify CD127 or S18 transcript copy numbers, CD127 and S18 PCR products were extracted using the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences). The number of mRNA copies/µl was calculated using the following formula: $[(6.022 \times 10^{23}) \times$ (concentration of DNA sample in micrograms/ µl)] / [(weight of 1 copy of mRNA) x (10^6 micrograms)], where the weight (g) of 1 copy of mRNA = (# base pairs in PCR product) x (325 g/mol). Serial dilutions ranging from 1×10^{10} to 1×10^2 mRNA copies/ µl of purified CD127 or S18 PCR products were included in PCR experiments to generate standard curves from which sample mRNA copy numbers were extrapolated using the iCycler software. The CD127 mRNA copy numbers for each sample were normalized to the mRNA copy numbers of the S18 gene using a two-step formula: 1) Normalization Factor for $individual_{(n)}$, $treatment_{(a)}$ = (average S18 copy number for all $individuals_{(1, 2, 3, ..., n)}$ for

treatment$_{(a)}$) / (observed S18 copy number for individual$_{(n)}$); 2) Actual CD127 mRNA copy number for individual$_{(n)}$, treatment$_{(a)}$ = (observed CD127 copy number for individual$_{(n)}$, treatment$_{(a)}$) x (Normalization Factor for individual$_{(n)}$, treatment$_{(a)}$).

2.8 Immunofluorescence Microscopy for CD127

2.8.1 Culturing Conditions

Purified $CD8^+$ T cells were culture with media or IL-7 (10 000 pg/ml) for 10, 20 minutes, 2, 4 and 24 hours. For the 10 and 20 minutes time points, samples were pre-treated for 1 hour at 37°C with or without lactocystine (20 μM), a proteosome inhibitor that prevents receptor internalization.

2.8.2 Staining for CD127 and CD132

All samples were fixed (2 X 10^5 /100μl) in IC Fixation Buffer (eBioscience) for 20 minutes at room temperature, washed in cold PBS $^+$ 1% Ab serum, and permeabilized with Perm 2 (BD) for 15 minutes at room temperature. Staining for CD127 was conducted as follows: 1) IgG1 mouse anti-human CD127 monoclonal antibody (R & D Systems, Minneapolis, MN USA) for 20 minutes at 4°C, 2) AlexaFluor 488-conjugated goat anti-mouse IgG (Invitrogen) for 20 minutes at 4°C. Staining for CD132 was conducted as follows: 1) rat anti-human CD132 (BD Pharmingen, San Diego USA) for 20 minutes at 4°C, 2) AlexaFluor 488-conjugated goat anti-rat IgG (H $^+$ L) (Invitrogen). Following each antibody incubation step, cells were washed with PBS $^+$ 1% AB serum. After staining, cells were centrifuged to a pellet, resuspended in 10 μl PBS, layered onto a

microscope slid and visualized using a Zeiss laser sectioning microscope using a magnification of 110 and a 2x zoom. Images were subsequently processed using Adobe Photoshop 7.0 (Adobe, USA) to adjust brightness and contrast and to generate single layered and overlaid images.

2.9 Western Blotting for CD127

2.9.1 Culturing Conditions and Harvesting of Supernatants

To determine if IL-7 induced the release of CD127, 6 x 10^6 $CD8^+$ T cells were plated into 6-well tissue culture plates and treated with or without IL-7 (10 000 pg/ml). Following 24 hours of culture at 37°C and 5% CO_2, plates were centrifuged and cell-free supernatants were collected and frozen at -80°C for future use. A soluble IL-7R secreting cell line, WI-26VA4 was cultured in the presence of IL-7 (10 000 pg/ml) and cell-free supernatants were collected and included in the Western blot analyses as a positive control.

2.9.2 Quantification of Protein Content in Supernatants

Total protein present in supernatants was quantified using the Pierce BSA assay kit following manufacturer's instructions (Pierce). In brief, 25 ul of each sample or appropriate diluted BSC standard (2000-25 ug/ml) were added to one well of a 96-well UV plate. To each well, 200 ul of a 50:1 mixture of BSA reagent A and BSA reagent B were added and incubated for 30 minutes at 37°C. The plate was read using a SpectroMAX 190 microplate reader at 562 nm wavelengths. Calculation of protein content in each sample against the standard curve was performed using the SoftMax Pro software.

2.9.3 SDS-Page

30 µg of supernatant from each condition was combined with 1 x loading buffer and heated at 90°C for 5 minutes. Samples were then loaded onto a 4-15% Tris-HCL-Ready Gel (BioRad). Following 1 hour electrophoresis at 100 V, proteins were blotted onto Polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Milipore). Transfer was conducted for 1 h at 100 V, after which time the membranes were blocked in 5% skim milk/PBS four times for 15 minutes with shaking. Membranes were stained with primary antibody overnight at 4°C with shaking and washed 5 times for 5 minutes with TBST, followed by secondary antibody for 1 hour at room temperature with shaking. Membranes were then washed once again five times for 5 minutes and stained with tertiary antibody. Proteins were detected by ECL chemiluminescence (Pierce Biotechnologies, Rockford, IL). Densitometry analysis of protein bands was performed using GeneTools Software (Syngene, Frederick, MD USA).

2.9.4 Soluble CD127 in Human Plasma Samples

To determine if soluble CD127 was present in human plasma in health and HIV disease, blood was collected from HIV^- and HIV^+ individuals not receiving anti-retroviral therapy and who provided informed consent. Blood was collected in heparin-containing tubes and plasma was collected following the first step of Ficoll-Paque gradient separation as described above. All samples were diluted 1:5 in 0.1% Triton X/PBS for 60 minutes at 37°C to kill any viral particles and then stored at -80°C. Samples were electrophoresed in a 6% SDS-PAGE gel under reducing conditions. A constant volume of plasma was loaded into

each well since total plasma protein concentrations did not vary significantly among individuals. Serial dilutions of recombinant human CD127-Fc chimera protein (R & D Systems) were included as positive controls, also diluted in 0.1% Triton X/PBS. Detection of CD127 protein by Western blot was conducted as described above.

2.10 IL-7R Functional Assays

2.10.1 CD127 Expression in Whole Blood

All research conducted using blood from human subjects was approved by the Ottawa Health Research Ethics Board. Blood was collected into heparin-containing tubes and 100 µl of blood was incubated with CD8-PC5 and CD127-PE monoclonal antibodies (5 µl each) (Beckman Coulter) for 30 minutes at room temperature in the dark. Samples were then processed by the Q-Prep/ImmunoPrep lysing system (Beckman Coulter), followed by flow cytometric analysis.

2.10.2 Preparation of cells

2.10.2.1 Peripheral Blood Mononuclear Cells (PBMCs)

As described in 2.2.2, PBMCs were isolated by Ficoll-Paque^{TM} PLUS (Pharmacia Fine Chemicals) gradient separation, washed and cultured ($1x10^6$ cells/ml) in complete RPMI Medium (Invitrogen) supplemented with 10% fetal calf serum (Cansera), 100 IU/ml each of penicillin and streptomycin (Sigma-Aldrich).

2.10.2.2 *Purified $CD8^+CD127^+$ T Cell*

First $CD8^+$ T cells were isolated from PBMCs using the human $CD8^+$ T cell isolation kit II following manufacturer's instructions (Miltenyi Biotec) along with the autoMACS cell sorter's "depletes" protocol to remove non-$CD8^+$ T-cells (Miltenyi Biotec). In brief, PBMCs were resuspended in 40 μl of MACS buffer and 10 μl of Biotin-Antibody cocktail per 10^7 total cells and incubated at 4°C. After 10 minutes, 30 μl of MACS buffer and 20 μl of Anti-Biotin Microbeads per 10^7 total cells was added and incubated for an additional 15 minutes at 4°C. Cells were then cultured ($1x10^6$ cells/ml) overnight in complete RPMI medium. The $CD127^+$ and $CD127^-$ cells were separated by labelling $CD8^+$ T cells with PE-conjugated mouse-anti-human CD127 monoclonal antibodies (65 μl per 10^7cells, Beckman Coulter) in 80 μl of MACS buffer per 10^7 cells and incubated for 20 minutes at 4°C. Cells were washed and resuspended in 80 μl of MACS buffer and 40 μl of Anti-PE MicroBeads (Miltenyi Biotec) per 10^7 cells for 15 minutes at 4°C. Cells were washed and separated using the autoMACS cell sorter's "possels" protocol to separate the antibody-labelled $CD127^+$ T cells from the $CD127^-$ T-cells. The $CD8^+$ T cell isolation typically yielded a > 90% pure population (data not shown), with minimal numbers of $CD4^+$ T cells and no contaminating monocytes. The separation of CD127 subsets typically enriched a $CD127^+$ population (>95% $CD127^+$) comparable to similar methods used to enrich other $CD8^+$ T cell subsets [104].

2.10.3 CFSE Labelling and T Cell Proliferation Assay

Following isolation, $CD8^+CD127^+$ cells were labelled with CFSE (CellTrace™ CFSE Cell Proliferation Kit) using a method modified from the manufacturer's specifications (Invitrogen Canada Inc., Burlington, ON Canada). A stock solution (5mM) of CFSE was prepared in DMSO followed by the preparation of a working solution (8μM) of CFSE in PBS + 0.1% BSA. Cells were resuspended in CFSE working solution ($1x10^7$ cells / ml) and incubated at 37°C, in the dark for 10 minutes. Cell were incubated with 15 volumes of cold complete RPMI on ice, in the dark for 5 minutes and then washed and resuspended in complete RPMI ($1x10^6$ cells / ml). Cells were cultured (1×10^5 cells/200 μl) in 96-well microtiter plates with medium only, PHA (0.5 μg/ml, Sigma-Aldrich), IL-7 (10 ng/ml, BD Biosciences, Mississauga, ON, Canada), or PHA (0.5 μg/ml) + IL-7 (10 ng/ml). The concentration of PHA used was the lowest tested concentration (i.e. sub-maximal dose) that induced at least 2 rounds of cell division among activated cells. Cells were cultured for 5 days and cell division was assessed by flow cytometry.

2.10.4 Intracellular Phosphorylated STAT5 Expression

Isolated $CD8^+CD127^+$ T cells were incubated (1×10^5 cells/200 μl) in 96-well microtiter plates with medium or IL-7 (1000 pg/ml) for 15 minutes. Cells were re-suspended in 100 μl of buffer reagent A - Fix (Invitrogen), incubated at room temperature for 15 minutes, and washed in PBS. Cells were re-suspended in 100 μl cold methanol, incubated at 4°C for 10 minutes and washed. Cells were then resuspended in buffer reagent B - Perm ($100\mu l/10^5$ cells, Invitrogen) with Alexa

Fluor® 488-conjugated anti-STAT5 antibody (5 µl/10^5 cells, BD Biosciences) and incubated at room temperature for 20 minutes. Samples were washed and analysis was performed by flow cytometry. The percentage of positive cells was measured from a cut-off set using an unstained control. Mean channel fluorescence (MFI) was measured over the entire lymphocyte population.

2.10.5 Expression of CD107a/b

Isolated $CD8^+CD127^+$ T cells were incubated (1 x 10^5 cells/200 µl) in 96-well microtiter plates with medium or IL-7 (10 000 pg/ml). Following 24 hours, cells were stained with 10 ul each CD107a and CD107b (BD Biosciences) for 1h at 37°C. Cells were re-suspended in fixation buffer reagent A - Fix (100µl/10^5 cells, Invitrogen), incubated at room temperature for 15 minutes and washed in PBS. Cells were then re-suspended in buffer reagent B - Perm (100µl/10^5 cells, Invitrogen) and incubated at room temperature for 20 minutes. Samples were washed and analysis was performed by flow cytometry. The percentage of positive cells was measured from a cut-off set using an unstained control. Mean channel fluorescence (MFI) was measured over the entire lymphocyte population

2.10.6 Intracellular Bcl-2 Expression

Isolated $CD8^+CD127^+$ T cells were incubated (1 x 10^5 cells/200 µl) in 96-well microtiter plates with medium or IL-7 (10 ng/ml) for 48 hours. Cells were re-suspended in fixation buffer reagent A - Fix (100µl/10^5 cells, Invitrogen), incubated at room temperature for 15 minutes and washed in PBS. Cells were then re-suspended in buffer reagent B - Perm (100µl/10^5 cells, Invitrogen) with FITC-conjugated Bcl-2 antibody (2 µl/10^5 cells, BD Biosciences) and incubated at

room temperature for 20 minutes. Samples were washed and analysis was performed by flow cytometry. The percentage of positive cells was measured from a cut-off set using an isotype control. Mean channel fluorescence (MFI) was measured over the entire lymphocyte population

2.10.7 Statistical Analysis

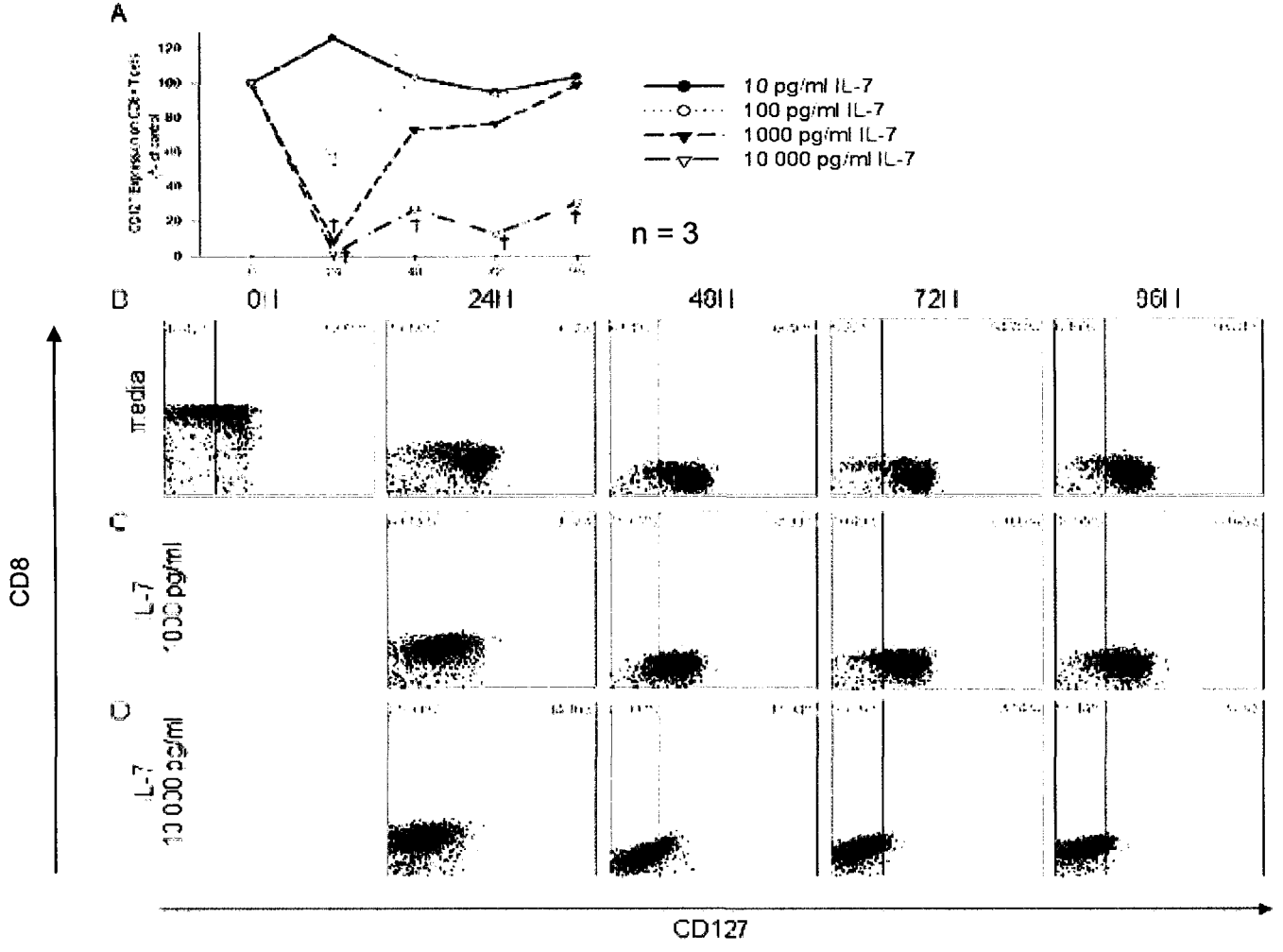
The Student's *t*-test or paired *t*-test was used for data analysis of as appropriate using PRISM where values of $p \leq 0.05$ were considered statistically significant.

3 RESULTS

3.1 Determining which Host or Viral Factors May be Responsible for the Donwregulation of CD127 Expression Observed in HIV Infection

3.1.1 IL-7 Decreases CD127 Expression on $CD8^+$ T Cells in PBMC Cell Cultures

A publication from this lab demonstrated that significantly fewer $CD8^+$ T cells express CD127 in HIV infected patients with uncontrolled viremia compared to healthy controls [83]. This report prompted an investigation of the mechanism(s) that decrease CD127 expression on $CD8^+$ T cells. Addition of IL-7 to PBMC cultures from healthy donors significantly decreased CD127 expression on $CD8^+$ T cells (FIGURE 1A). This decrease occurred mainly on $CD8^+CD45RA^+$ T cells. With concentrations of IL-7 up to 1000 pg/ ml, this occurred in a transient nature (FIGURE 1A, C) but was sustained with higher concentrations (10 000 pg /ml) (FIGURE 1A, D). Based on four-color flow cytometry analysis, CD127 expression on $CD45RA^+$ cells occurs almost exclusively on $CD45RA^+CD27^+$ (FIGURE 2) or $CD45RA^+CD62L^+$ cells (truly naïve cells) (data not shown). Gating on $CD45RA^+$ alone therefore reflects an accurate evaluation of the expression of CD127 on naïve cells without requiring the use of additional cell-surface markers. Of note, incubation of IL-7 did not affect the proportion of $CD8^+$ T cells that expressed CD45RA or CD45R0 (data not shown). As previously described (22), expression of CD127 on $CD8^+$ T cells in PBMCs recovers after 24 hours in culture and remains stable over time (FIGURE 1B).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100 pg/ml IL-7
1000 pg/ml IL-7
10 000 pg/ml IL-7
n = 3
B
0 H
24 H
48 H
72 H
96 H
media
C
L-7
1000 pg/ml
D
L-7
10 000 pg/ml
CD8
CD127

FIGURE 1: Effect of IL-7 on CD127 expression on $CD8^+$ T cells within PBMC cultures. (A) Incubation of PBMCs with increasing concentrations of IL-7 (100–1000 pg/ml) resulted in statistically significant yet transient decreases of CD127 expression on $CD8^+$ T cells in PBMC cultures ($n = 3$, $^*P = 0.001$; $^yP < 0.001$ by analysis of variance and $P < 0.05$ by Dunnett's simultaneous test versus time 0). A sustained decrease in CD127 expression was observed following incubation with 10 000 pg/ml of IL-7. Representative dot plots of CD127 expression on $CD8^+$ T cells in PBMCs cultured over time are also shown. (B) Expression of CD127 recovers after 24 hours in medium alone and is sustained over time. (C) Incubation with IL-7 (1000 pg/ml) resulted in a transient decrease of CD127 expression. (D) High concentrations of IL-7 (10 000 pg/ml) resulted in a sustained down-regulation of CD127.

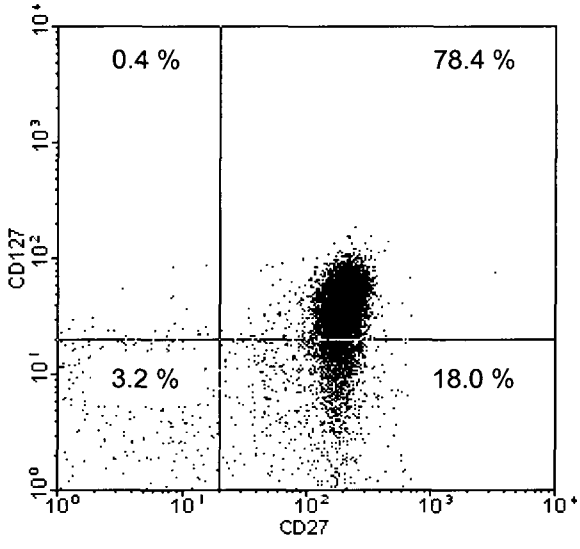

0.4 %
78.4 %
3.2 %
18.0 %
CD127
CD27
10⁰
10¹
10²
10³
10⁴

FIGURE 2: Gating on CD45RA$^+$ cells reflects an accurate evaluation of the expression of CD127 on naïve cells. Based on four-color flow cytometric analysis and gating on the CD3$^+$CD8$^+$CD45RA$^+$ T cell populations, CD127 expression on CD45RA$^+$ cells occurs almost exclusively (78.4% / [78.4% $^+$ 0.4%] > 99%) on the CD45RA$^+$CD27$^+$ population (truly naïve cells).

Analysis of mean channel fluorescence (MCF) was also performed in parallel and IL-7 stimulation resulted in significant decreases in MCF (data not show).

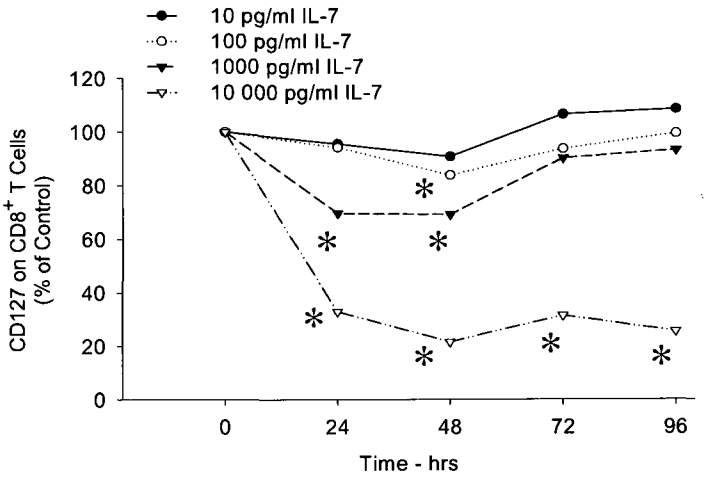
3.1.2 IL-7 Decreases CD127 Expression on Purified $CD8^+$ T Cells, Mainly on Naïve $CD8^+$ T Cells

To determine if IL-7 acts directly on $CD8^+$ T cells, purified $CD8^+$ T cells were cultured with increasing concentrations of the cytokine. The expression of CD127 on $CD8^+$ T cells was transiently decreased by IL-7 (100-1000 pg/ml) and returned to baseline levels after 96 hours of incubation (FIGURE 3A). Occurring almost exclusively on $CD8^+CD45RA^+$ T cells (FIGURE 3B and C), this effect was detected within the first 24 hours and was sustained for 96 hours on both $CD45RA^+$ and $CD45RO^+$ subpopulations with 10 000 pg/ml of IL-7.

3.1.3 IL-4 Decreases CD127 Expression on $CD8^+$ T Cells in PBMC cultures

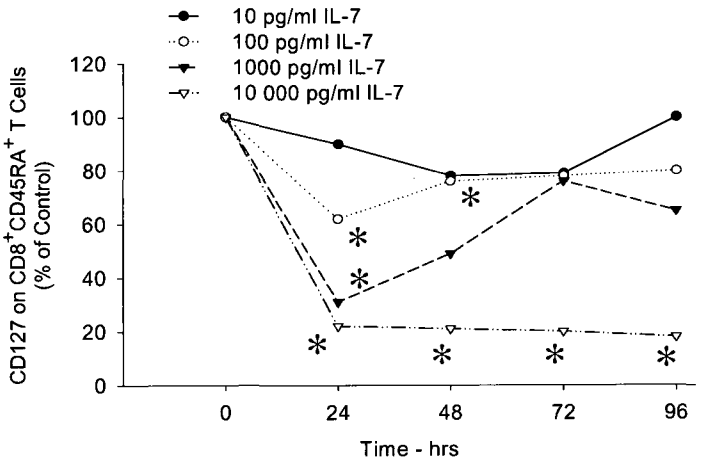
Addition of IL-4 significantly decreased CD127 expression on $CD8^+$ T cells in PBMC cultures (FIGURE 4A). This down-regulation occurred exclusively on $CD8^+CD45RA^+$ T cells (FIGURE 4B), while no significant changes were detected on $CD8^+CD45RO^+$ cells (FIGURE 4C). Of note, incubation of IL-4 did not affect the proportion of $CD8^+$ T cells that expressed CD45RA or CD45R0.

A


10 pg/ml IL-7
100 pg/ml IL-7
1000 pg/ml IL-7
10 000 pg/ml IL-7
CD127 on CD8+ T Cells (% of Control)
120
100
80
60
40
20
0
0
24
48
72
96
Time - hrs

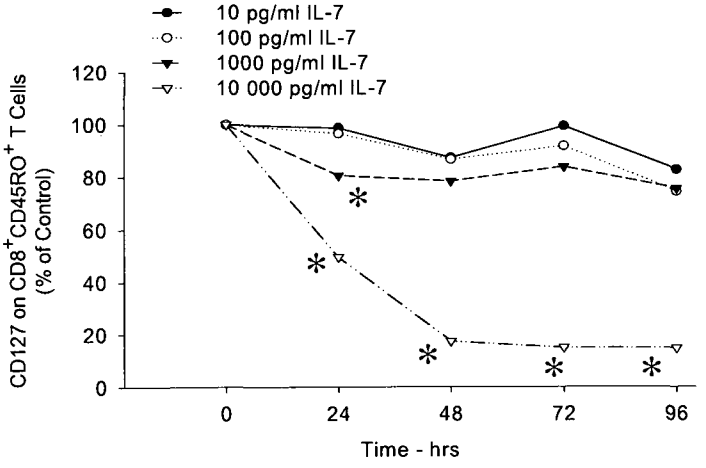
n = 3

B


10 pg/ml IL-7
100 pg/ml IL-7
1000 pg/ml IL-7
10 000 pg/ml IL-7
CD127 on CD8+CD45RA+ T Cells (% of Control)
120
100
80
60
40
20
0
0
24
48
72
96
Time - hrs

n = 3

C


10 pg/ml IL-7
100 pg/ml IL-7
1000 pg/ml IL-7
10 000 pg/ml IL-7
CD127 on CD8+CD45RO+ T Cells (% of Control)
120
100
80
60
40
20
0
0
24
48
72
96
Time - hrs

n = 3

FIGURE 3: IL-7 decreases surface CD127 expression in purified $CD8^+$ T cell cultures. (A) The expression of CD127 in purified $CD8^+$ T cells was transiently decreased by IL-7 (100–1000 pg/ml) which returned to baseline levels by 96 hours of incubation. The highest concentration of IL-7 (10 000 pg /ml) resulted in a sustained decrease in CD127 expression (n = 3, *P < 0.001 and yP = 0.013 by analysis of variance (ANOVA) and P < 0.05 by Dunnett's simultaneous test versus time 0). To determine if this effect occurred on a specific sub-population of $CD8^+$ T cells, the expression of CD127 on $CD45RA^+$ (B) and $CD45RO^+$ (C) $CD8^+$ T cells was assessed. Treatment with IL-7 (100–1000 pg/ml) transiently decreased CD127 expression almost exclusively on $CD8^+CD45RA^+$ T cells (B), which returned to baseline after 96 h. The highest concentration of IL-7 (10 000 pg /ml) resulted in a sustained decrease of CD127 on both $CD45RA^+$ and $CD45RO^+$ sub-populations (C) (n = 3, *P = 0.020; yP = 0.017 and zP < 0.001 by ANOVA or ANOVA on ranks as appropriate and P < 0.05 by Dunnett's simultaneous test versus time 0).

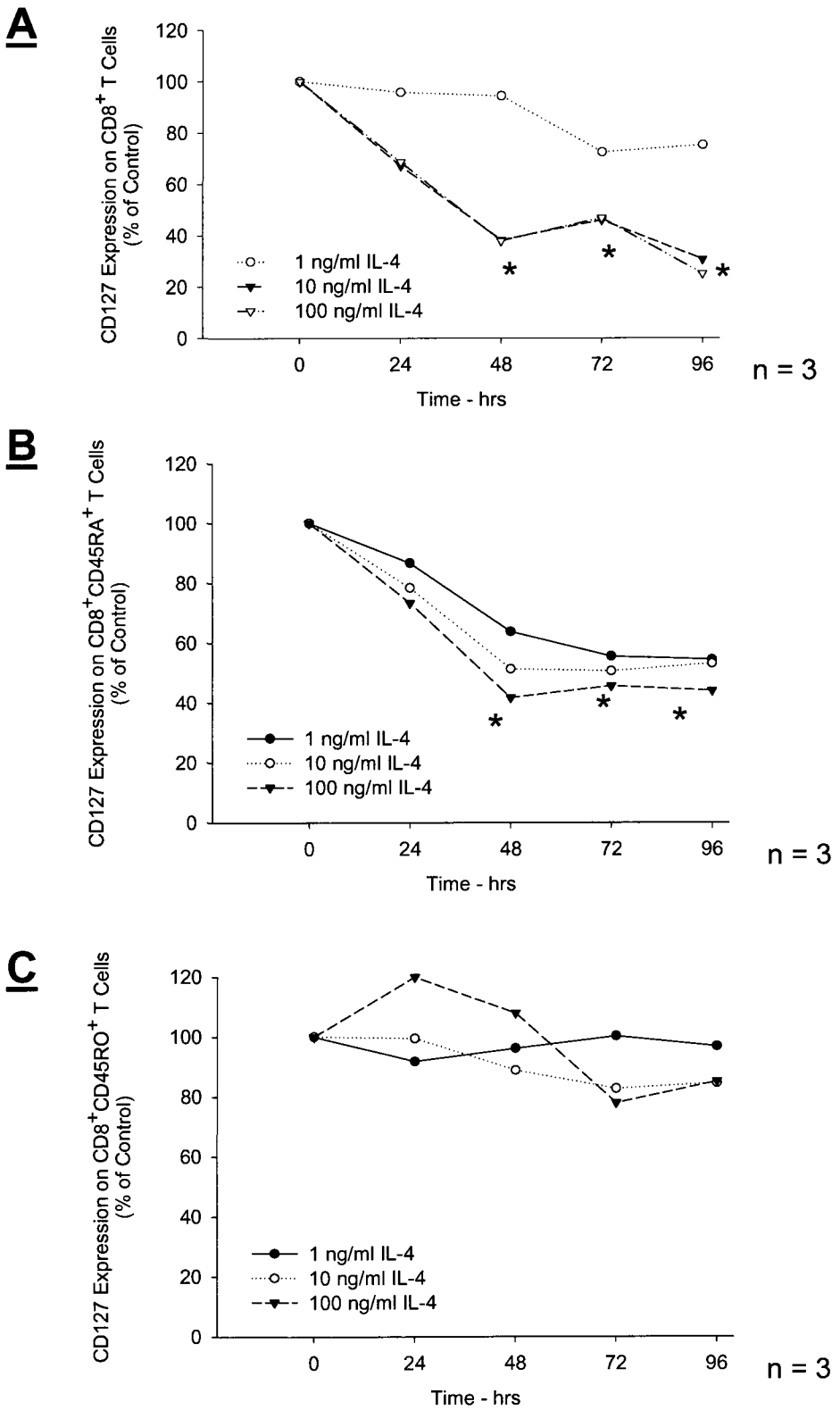

A
CD127 Expression on CD8+ T Cells (% of Control)
120
100
80
60
40
20
0
1 ng/ml IL-4
10 ng/ml IL-4
100 ng/ml IL-4
*
*
*
0
24
48
72
96
Time - hrs
n = 3
B
CD127 Expression on CD8+CD45RA+ T Cells (% of Control)
120
100
80
60
40
20
0
1 ng/ml IL-4
10 ng/ml IL-4
100 ng/ml IL-4
*
*
*
0
24
48
72
96
Time - hrs
n = 3
C
CD127 Expression on CD8+CD45RO+ T Cells (% of Control)
120
100
80
60
40
20
0
1 ng/ml IL-4
10 ng/ml IL-4
100 ng/ml IL-4
0
24
48
72
96
Time - hrs
n = 3

FIGURE 4: **IL-4 decreases surface CD127 expression on $CD8^+$ T cells in PBMC cultures.** (A) The highest concentration of IL-7 (1000 pg/ml) slightly decreased CD127 at 24 hours, although not significantly. (B) Increasing concentrations of IL-4 (1-100 ng/ml decreased surface CD127 expression on $CD8^+$ T cells in PBMC cultures (n = 3, *p = 0.030; †p = 0.013 by ANOVA). (C) To determine if CD127 down-regulation occurred on a specific subpopulation of $CD8^+$ T cells, cells were stained for expression of CD45RA (left panel) or CD45RO (right panel). The decrease in CD127 occurred mainly on $CD8^+CD45RA^+$ T cells. (n = 3, *p = 0.014; †p = <0.001 by ANOVA). In all cases, these effects were significant ($p<0.05$) over time as determined by Dunnett multiple comparison versus time 0.

3.1.4 IL-4 Decreases CD127 Expression on Purified $CD8^+$ T Cells, Mainly on Naïve $CD8^+$ T Cells

In purified $CD8^+$ T cells cultures, IL-4 (1-100 ng/ml) significantly decreased CD127 expression on $CD8^+$ T cells (FIGURE 5A). Similar to what was observed in PBMCs, IL-4 down-regulated CD127 expression on $CD8^+CD45RA^+$ T cells (FIGURE 5B), while no significant changes were detected on $CD8^+CD45R0^+$ cells (FIGURE 5C).

3.1.5 HIV-1 tat Decreases CD127 Expression on Purified $CD8^+$ T Cells

Addition of HIV-1 tat protein (1-1000 ng/ml) to purified $CD8^+$ T cells cultures decreased surface CD127 expression on $CD8^+$ T cells (FIGURE 6). This effect seems to be time and dose dependent.

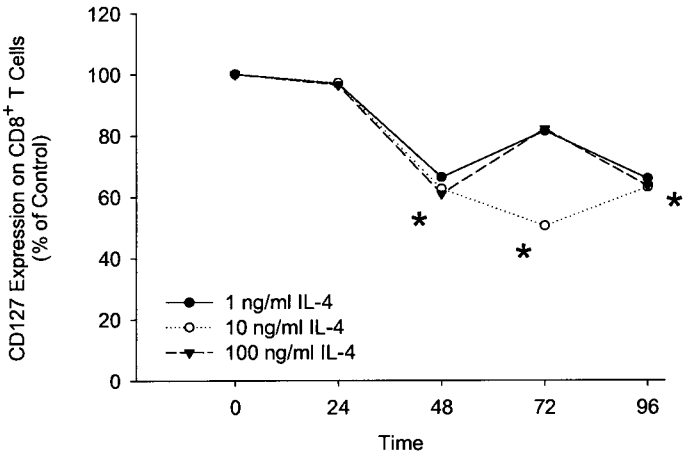
3.1.6 Changes in CD127 Expression Are not Due to Mortality

To exclude IL-7 or IL-4-induced selective mortality as a potential contributor to the observed results, PI staining was performed on PBMCs and purified $CD8^+$ T cells incubated with IL-7 (1000 pg/ml) or IL-4 (100 ng/ml) for 24, 48, 72 and 96 hours, and no significant cell mortality was detected (FIGURE 7).

3.1.7 HIV-1 gp120, IL-1-β, IL-6, IL-10, IL-13, TGF-β or TNF-α Have no Effect on CD127 Expression

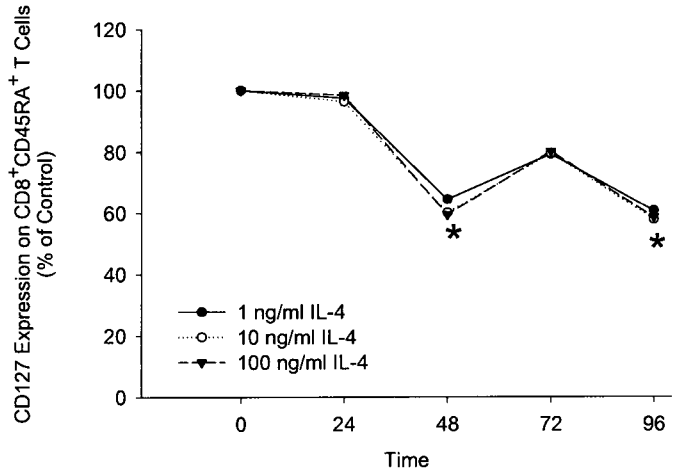
No significant change in CD127 expression on $CD8^+$ T cells in PBMC cultures as well as purified $CD8^+$ T cells was observed following incubation with

A


CD127 Expression on CD8+ T Cells
(% of Control)
120
100
80
60
40
20
0
0
24
48
72
96
Time
1 ng/ml IL-4
10 ng/ml IL-4
100 ng/ml IL-4
*
*
*

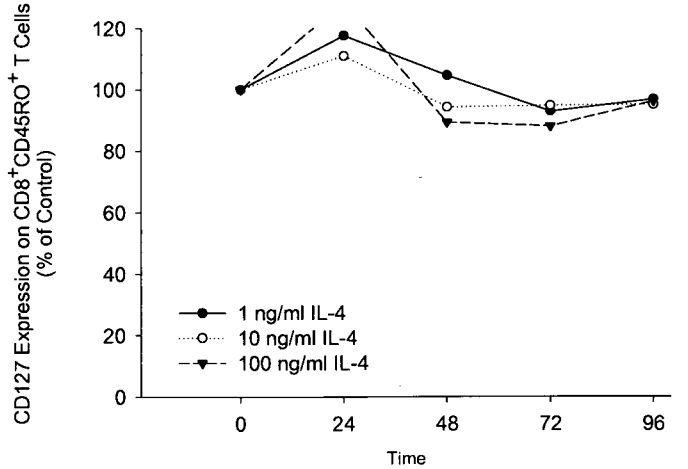
n = 3

B


CD127 Expression on CD8+CD45RA+ T Cells
(% of Control)
120
100
80
60
40
20
0
0
24
48
72
96
Time
1 ng/ml IL-4
10 ng/ml IL-4
100 ng/ml IL-4
*
*

n = 3

C


CD127 Expression on CD8+CD45RO+ T Cells
(% of Control)
120
100
80
60
40
20
0
0
24
48
72
96
Time
1 ng/ml IL-4
10 ng/ml IL-4
100 ng/ml IL-4

n = 3

FIGURE 5: **IL-4 decrease surface CD127 expression in purified $CD8^+$ T cell cultures.** (A) Treatment of purified $CD8^+$ T cells with increasing concentrations of IL-4 (1-100 ng/ml) resulted in a significant decrease in surface CD127 expression in a time- and dose-dependent manner (n = 3, *p = 0.006; †p = <0.001 by ANOVA). To determine if CD127 down-regulation occurred on a specific subpopulation of $CD8^+$ T cells, cells were also stained for expression of CD45RA (upper panel) or CD45RO (lower panel). (B)(C) Increasing concentrations of IL-4 (1-100 ng/ml) decreased CD127 expression exclusively on the CD45RA subpopulation of $CD8^+$ T cells (n = 3, *p = 0.017; †p = <0.001; ‡p = 0.015 by ANOVA or ANOVA on ranks as appropriate.) In all cases, these effects were significant over time ($p<0.05$) as determined by Dunnett's multiple comparison versus time 0).

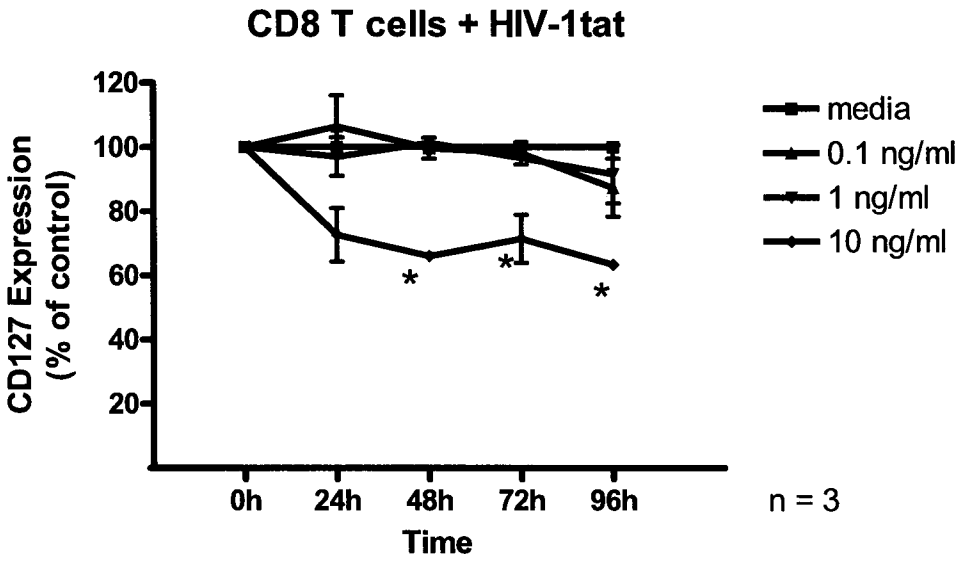

CD8 T cells + HIV-1tat
CD127 Expression (% of control)
120
100
80
60
40
20
0h
24h
48h
72h
96h
Time
media
0.1 ng/ml
1 ng/ml
10 ng/ml
*
*
*
n = 3

FIGURE 6: HIV-1tat decrease surface CD127 expression in purified $CD8^+$ T cell cultures. (A) Treatment of purified $CD8^+$ T cells with increasing concentrations of HIV-1 tat (0.1-10 μg/ml) resulted in a significant decrease in surface CD127 expression. ($n = 3$, $^*p < 0.05$ by ANOVA).

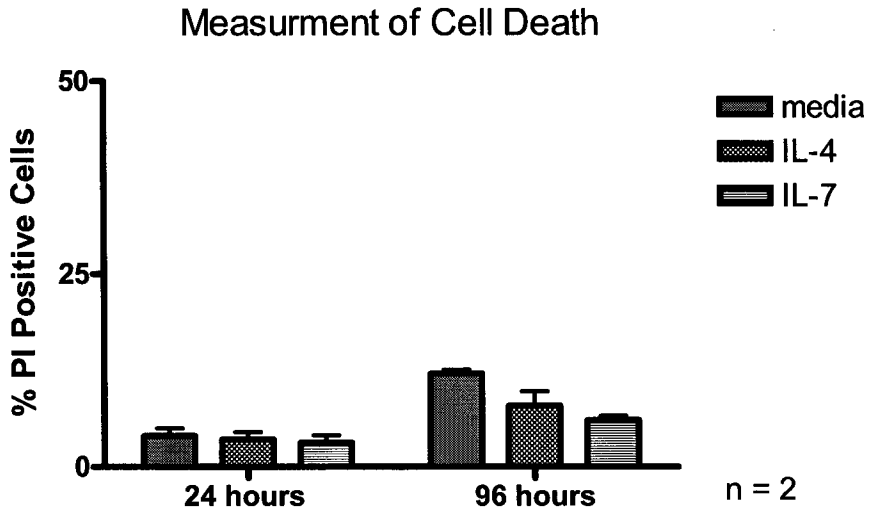

Measurment of Cell Death
% PI Positive Cells
50
25
0
24 hours
96 hours
media
IL-4
IL-7
n = 2

FIGURE 7: Down-regulation in surface CD127 expression is not due to cell death. Purified $CD8^+$ T cells were cultured in media alone or with IL-4 (100 ng/ml) or IL-7 (1000 ng/ml). At 24 and 96 hours, addition of IL-4 or IL-7 did not cause an increase in cells death. (n = 2)

IL-1β (FIGURE 8A), IL-6 (FIGURE 8B), IL-10 (FIGURE 8C), IL-13 (FIGURE 8D), TGF-β (FIGURE 9A), IFN-α (FIGURE 9B), TNF-α (FIGURE 9C), HIV-1 gp120 (FIGURE 10A) and HIV-1 nef (FIGURE 10B).

3.2 Determining the Mechanism by which Host or Viral Factors Decrease CD127 Expression on $CD8^+$ T Cells

3.2.1 CD127 RNA Expression is Unchanged by IL-7, IL-4 and HIV-1 tat

A semi-quantitative PCR method was developed to detect expression of mRNA that encoded membrane-bound CD127 and results were confirmed by quantitative real-time PCR. Treating PBMCs with IL-7 (10 000 pg/ml) for 8, 16 and 24 hours did not alter expression of mRNA encoding the membrane form of CD127. Treating purified $CD8^+$ T cells with IL-7 (10 000 pg/ml) (FIGURE 11A), IL-4 (100 ng/ml) (FIGURE12A) or HIV-1 tat (10 ug/ml) (FIGURE 13) for 16 or 24 hours did not alter the expression of mRNA encoding the membrane form of CD127. For IL-7 and IL-4, these observations were confirmed by quantitative real-time PCR, in which IL-7 (FIGURE 11B) and IL-4 (FIGURE 12B) did not decrease the expression of CD127 mRNA from $CD8^+$ T cells.

3.2.2 IL-7 Does not Increase Cytokine Receptor Internalization

Since IL-7 transiently decreases CD127 expression on $CD8^+$ T cells without affecting CD127 mRNA expression, other mechanisms may be involved in surface receptor down-regulation such as receptor internalization. After culture with media or IL-7 (10 000 pg/ml) for 30 minutes to 24 hours, cells were fixed and

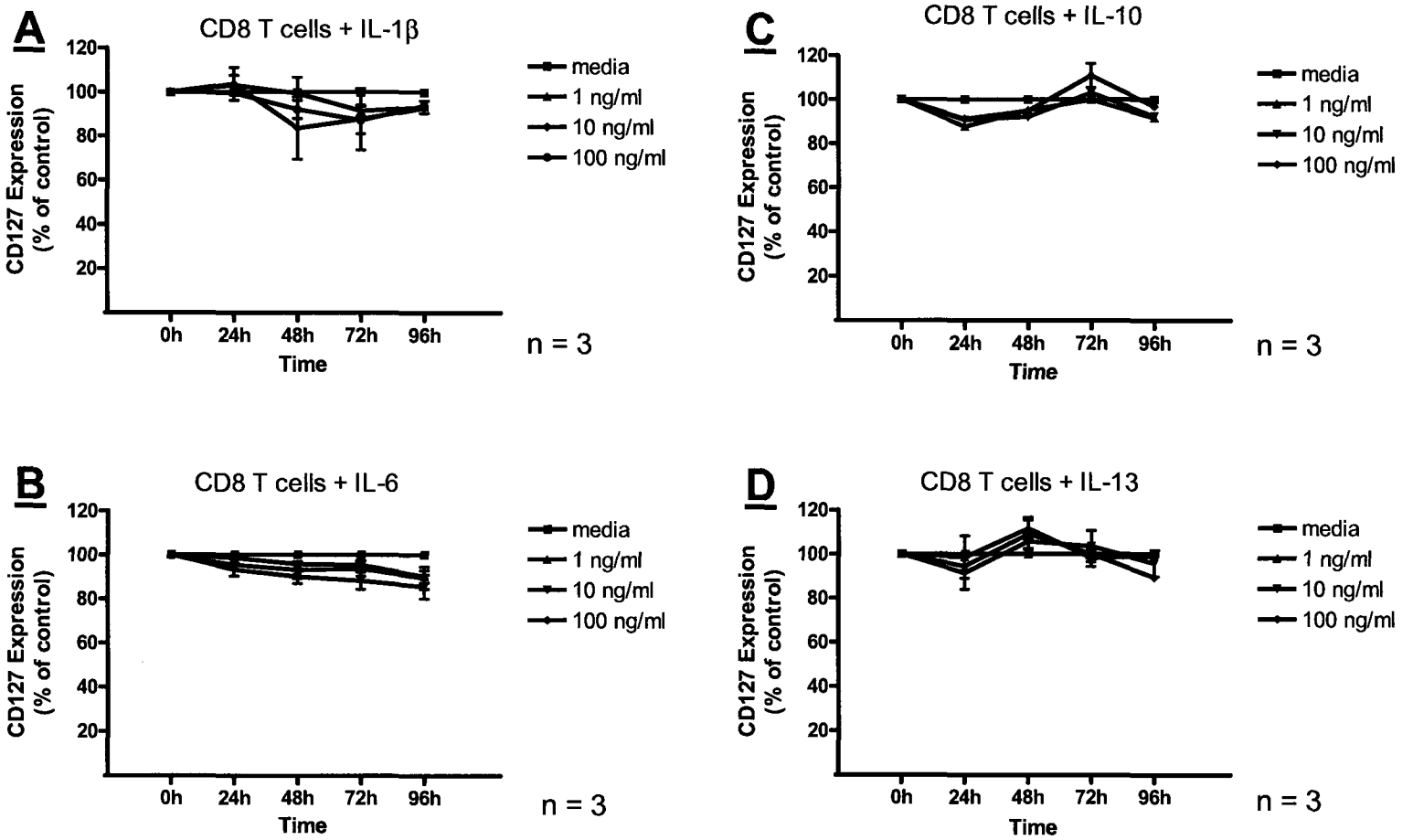
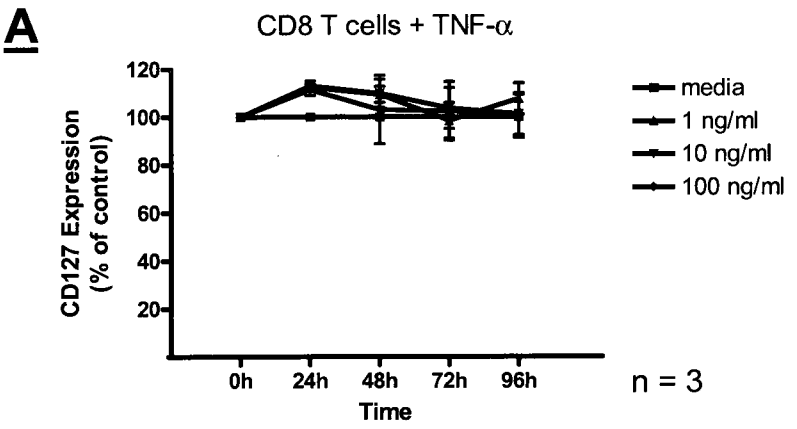
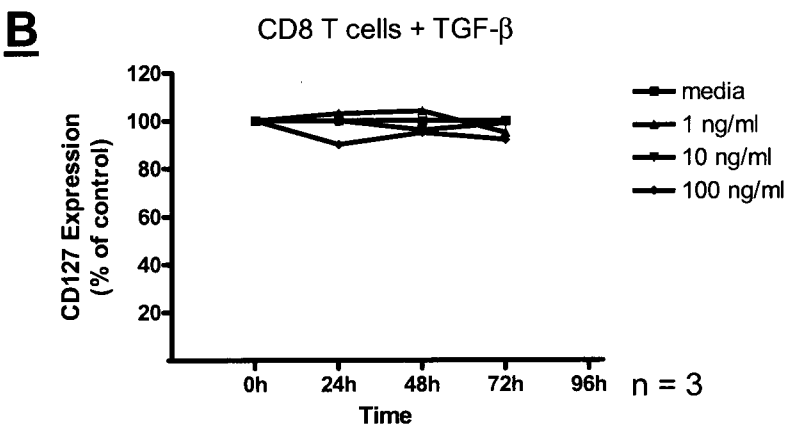

A
CD8 T cells + IL-1β
CD127 Expression (% of control)
120
100
80
60
40
20
0h
24h
48h
72h
96h
Time
media
1 ng/ml
10 ng/ml
100 ng/ml
n = 3
C
CD8 T cells + IL-10
CD127 Expression (% of control)
Time
media
1 ng/ml
10 ng/ml
100 ng/ml
n = 3
B
CD8 T cells + IL-6
CD127 Expression (% of control)
Time
media
1 ng/ml
10 ng/ml
100 ng/ml
n = 3
D
CD8 T cells + IL-13
CD127 Expression (% of control)
Time
media
1 ng/ml
10 ng/ml
100 ng/ml
n = 3

FIGURE 8: **IL-1β, IL-6, IL-10 and IL-13 have no effect on the expression of surface CD127 on $CD8^+$ T cells.** Treatment of purified $CD8^+$ T cells with increasing concentrations of (A) IL-1β (1-100 ng/ml), (B) IL-6 (1-100 ng/ml), (C) IL-10 (1-100 ng/ml) or (D) IL-13 (1-100 ng/ml) resulted in no significant decrease in surface CD127 expression (n=3)


A
CD8 T cells + TNF-α
CD127 Expression (% of control)
120
100
80
60
40
20
0h
24h
48h
72h
96h
Time
media
1 ng/ml
10 ng/ml
100 ng/ml
n = 3


B
CD8 T cells + TGF-β
CD127 Expression (% of control)
120
100
80
60
40
20
0h
24h
48h
72h
96h
Time
media
1 ng/ml
10 ng/ml
100 ng/ml
n = 3

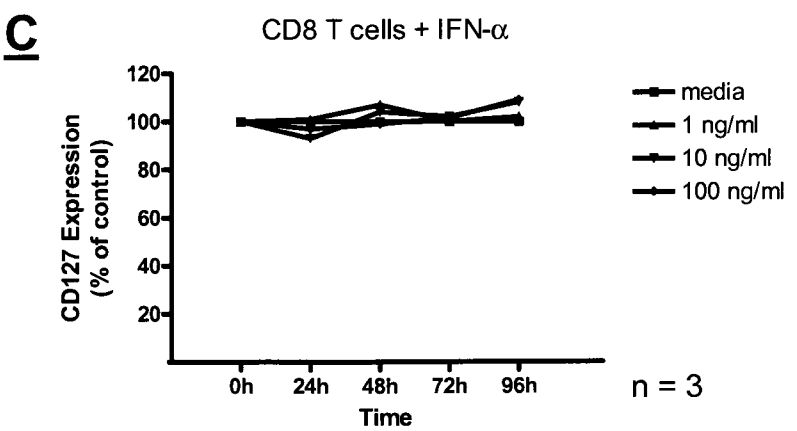
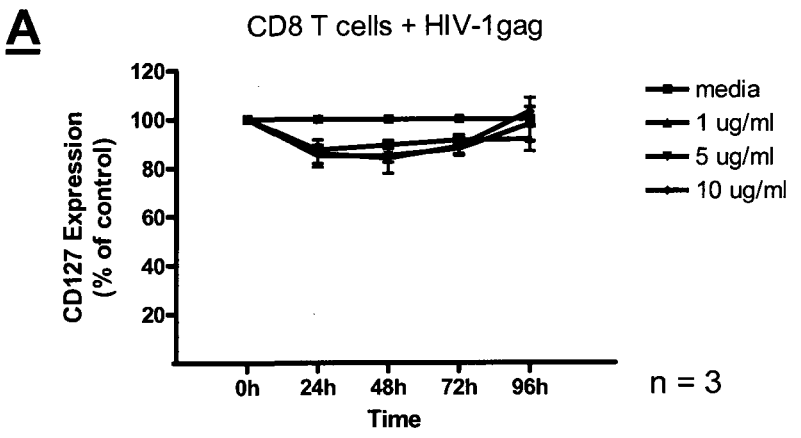

C
CD8 T cells + IFN-α
CD127 Expression (% of control)
120
100
80
60
40
20
0h
24h
48h
72h
96h
Time
media
1 ng/ml
10 ng/ml
100 ng/ml
n = 3

FIGURE 9: TGF-β, IFN-α and TNF-α have no effect on the expression of surface CD127 on $CD8^+$ T cells. Treatment of purified $CD8^+$ T cells with increasing concentrations of (A) TGF-β (1-100 ng/ml), (B) IFN-α (1-100 ng/ml) or (C) TNF-α (1-100 ng/ml) resulted in no significant decrease in surface CD127 expression (n=3)


A
CD8 T cells + HIV-1gag
CD127 Expression (% of control)
120
100
80
60
40
20
0h
24h
48h
72h
96h
Time
media
1 ug/ml
5 ug/ml
10 ug/ml
n = 3

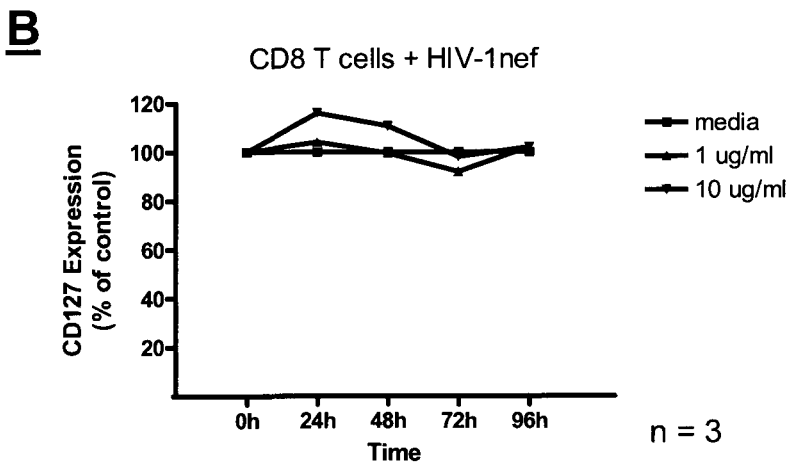

B
CD8 T cells + HIV-1nef
CD127 Expression (% of control)
120
100
80
60
40
20
0h
24h
48h
72h
96h
Time
media
1 ug/ml
10 ug/ml
n = 3

FIGURE 10: **HIV-1 gp120 and HIV-1 nef proteins have no effect on the expression of surface CD127 on $CD8^+$ T cells.** Treatment of purified $CD8^+$ T cells with increasing concentrations of (A) HIV-1 gag (1-10 µg/ml) or (B) HIV-1 nef (1-10µg/ml) resulted in no significant decrease in surface CD127 expression (n=3)

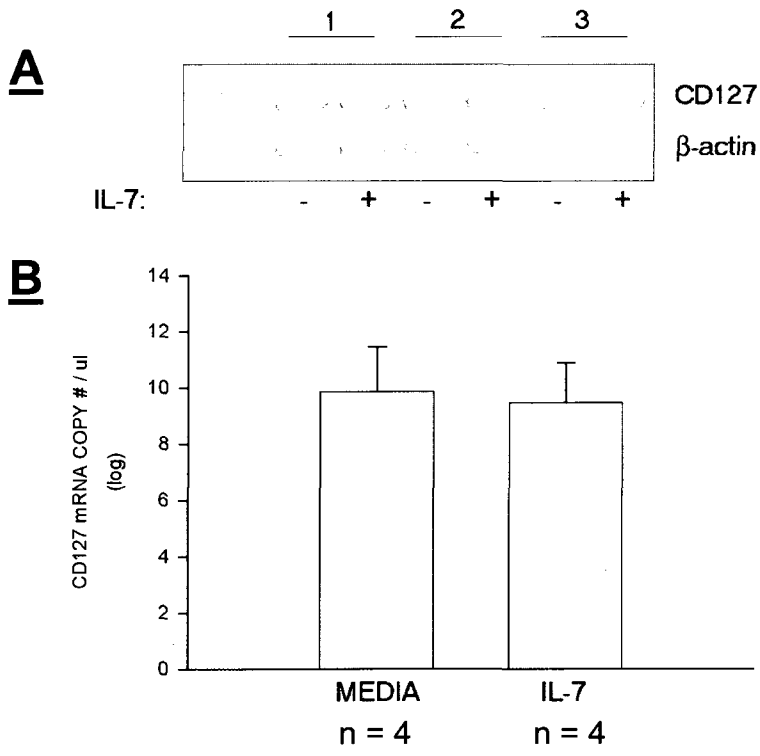

A
1
2
3
CD127
β-actin
IL-7:
-
+
-
+
-
+
B
CD127 mRNA COPY # / ul
(log)
14
12
10
8
6
4
2
0
MEDIA
IL-7
n = 4
n = 4

FIGURE 11: **IL-7 has no effect on the expression of mRNA-encoding membrane-bound CD127 mRNA in $CD8^+$ T cells.** The effect of IL-7 on CD127 gene expression was studied to further evaluate the mechanism of CD127 down-regulation on $CD8^+$ T cells. (A) Addition of IL-7 (10 000 pg/ ml) for 24 hours did not alter the amount of mRNA encoding membrane-bound CD127 expressed by $CD8^+$ T cells as determined by semi-quantitative PCR (n = 3). The upper bands represent the expression of CD127 and the lower bands represent the expression of the beta-actin housekeeping gene. The molecular weight marker is shown on the left. (B) The lack of change in the expression of mRNA encoding membrane-bound CD127 mRNA in IL-7-stimulated $CD8^+$ T cells was confirmed by real-time PCR (n = 4). The expression of CD127 mRNA was normalized to the expression of the ribosomal subunit-18.

A

CD127

IL-4: - +

B

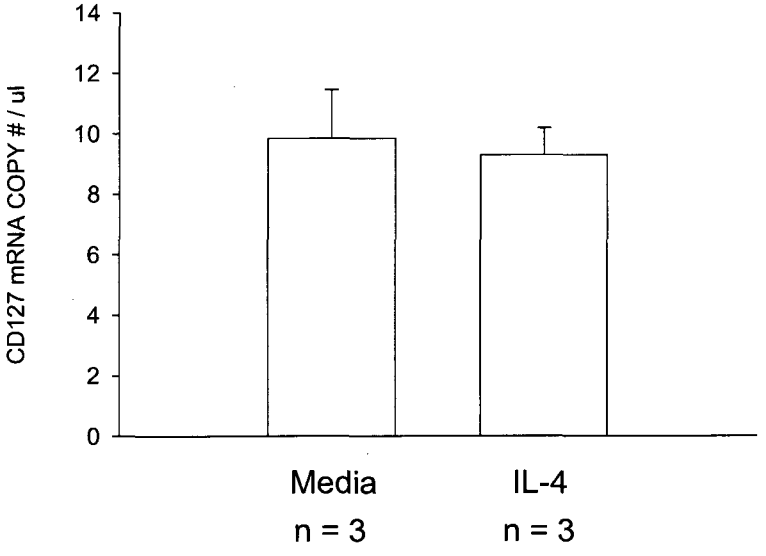


FIGURE 12: IL-4 has no effect on the expression of mRNA-encoding membrane-bound CD127 mRNA in $CD8^+$ T cells. The effect of IL-4 on CD127 gene expression was studied to further evaluate the mechanism of CD127 down-regulation on $CD8^+$ T cells. (A) Addition of IL-4 (100 ng/ml) for 24 hours did not alter the amount of mRNA encoding membrane-bound CD127 expressed by $CD8^+$ T cells as determined by semi-quantitative PCR. The molecular weight marker is shown on the left. (one representation of three) (B) The lack of change in the expression of mRNA encoding membrane-bound CD127 mRNA in IL-4-stimulated $CD8^+$ T cells was confirmed by real-time PCR (n = 3). The expression of CD127 mRNA was normalized to the expression of the ribosomal subunit-18.

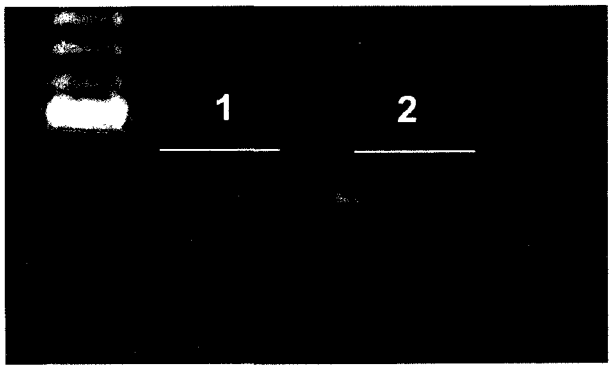

1
2
CD127
HIV-1 tat: - + - +

FIGURE 13: **HIV-1 tat has no effect on the expression of mRNA-encoding membrane-bound CD127 mRNA in $CD8^+$ T cells.** The effect of HIV-1 tat on CD127 gene expression was studied to further evaluate the mechanism of CD127 down-regulation on $CD8^+$ T cells. (A) Addition of HIV-1 tat (10 μg/ml) for 24 hours did not alter the amount of mRNA encoding membrane-bound CD127 expressed by $CD8^+$ T cells as determined by semi-quantitative PCR. The molecular weight marker is shown on the left (two representations of three).

permeabilized and cell surface and cytoplasmic CD127 were stained with anti-CD127 monoclonal antibodies. The effect of IL-2 on CD132 expression was used as a positive control for receptor internalization [105, 106]. As expected, IL-2 increased cytoplasmic CD132 (FIGURE14A). In contrast to IL-2, we did not observe an increase in CD127 within the cytoplasm of IL-7-stimulated cells (FIGURE 14B-D). Interleukin-7 consistently decreased the expression of surface CD127 on $CD8^+$ T cells compared to media controls in fixed-only or fixed and permeabilized cells (FIGURE 14E), confirming the results obtained by flow cytometry (FIGURE 1) . Preliminary results of IL-7-stimulated $CD8^+$ T cells pre-incubated with lactacystin, a known proteosome inhibitor, did not reveal an increase in cytosolic CD127 suggesting that proteosomal degradation does not account for the observed lack of internalized CD127 (FIGURE 15)

3.2.3 IL-7 Induces CD127 Shedding

An alternative mechanism by which IL-7 may down-regulate CD127 expression is via receptor shedding. Western blot analysis of supernatants collected from purified $CD8^+$ T cells after 24 hours of culture detected the presence of CD127 (FIGURE 16). In 3 out of 7 healthy donors, IL-7 (10 000 pg/ml) increased the amount of CD127 detected compared to medium only. In 2 out of 7 samples, although undetected in medium controls, CD127 was detected when the cells were cultured in the presence of IL-7 (10 000 pg/ml). In the remaining samples, no CD127 was detected in either control or IL-7-treated cells.

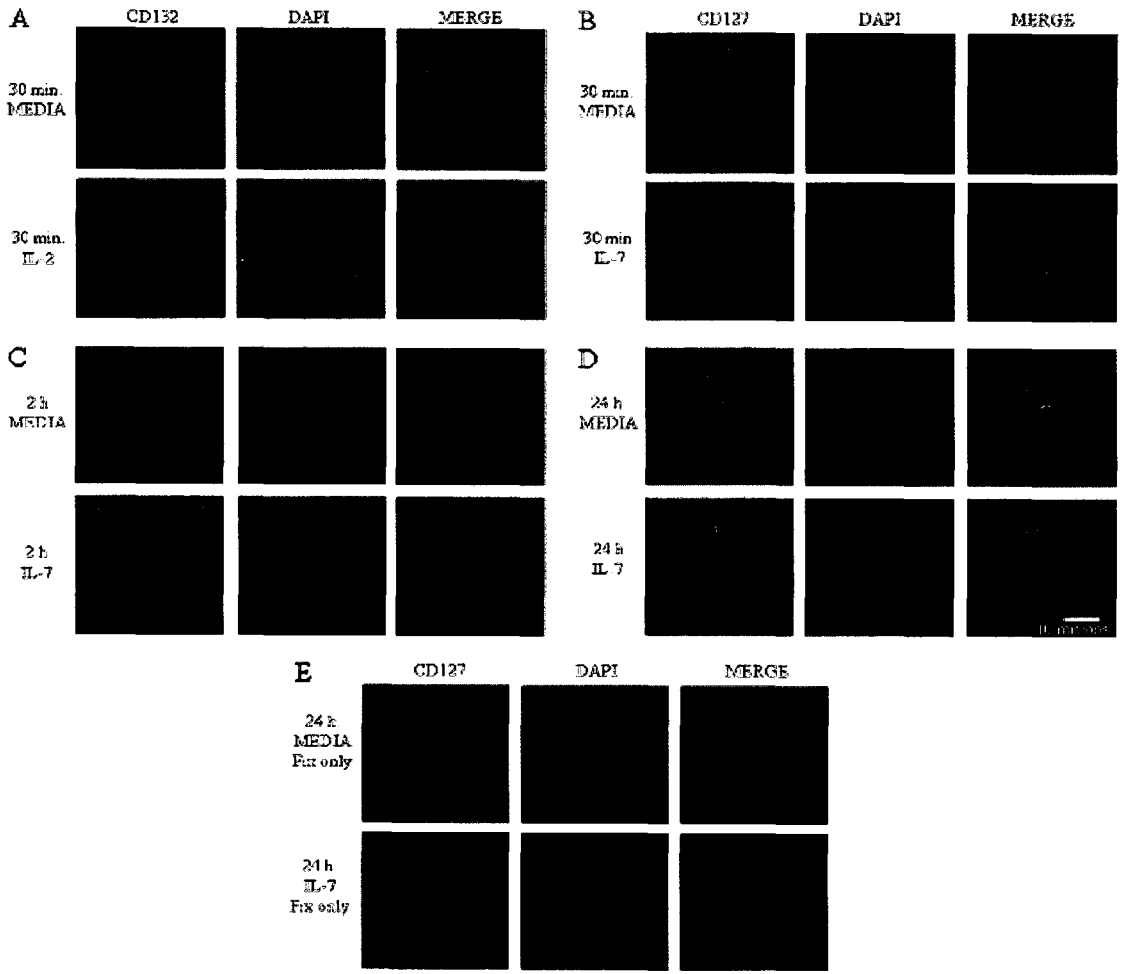
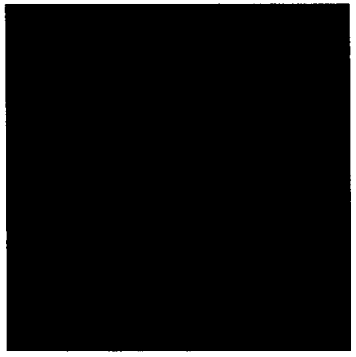

A
CD132
DAPI
MERGE
30 min.
MEDIA
30 min.
IL-2
B
CD127
DAPI
MERGE
30 min.
MEDIA
30 min
IL-7
C
2 h
MEDIA
2 h
IL-7
D
24 h
MEDIA
24 h
IL-7
E
CD127
DAPI
MERGE
24 h
MEDIA
Fix only
24 h
IL-7
Fix only

FIGURE 14: IL-7 does not significantly increase cytoplasmic CD127 in $CD8^+$ T cells. (A) As a positive control for the assessment of receptor internalization by confocal microscopy, the presence of cell and cytoplasmic CD132 were detected in fixed and permeabilized cells $CD8^+$ T cells that were incubated with IL-2 for 30 minutes. (B) Untreated $CD8^+$ T cells express abundant CD127 molecules on their cell surface after 30 minutes of culture and this remained unchanged in the presence of IL-7 (10 000 pg/ml). (C) Similarly, IL-7 did not increase the presence of cytoplasmic CD127 after 2 hours of culture. (D) Following 24 hours of culture, the expression of surface CD127 on $CD8^+$ T cells decreased significantly in response to IL-7, consistent with flow cytometry observations (Figure 3), while the amount of cytoplasmic CD127 remained unchanged. Therefore, CD127 does not accumulate within the cytoplasm, suggesting that IL-7 does not induce receptor internalization. Samples were visualized using a Zeiss laser-sectioning microscope using a magnification of 110 and a 2x zoom. Images were subsequently processed using Adobe Photoshop 7.0 (Adobe, USA) to adjust sharpeness, brightness, and contrast and to generate single layered and overlayed images. The image scale shown here is ≈ 10 microns.

A



B

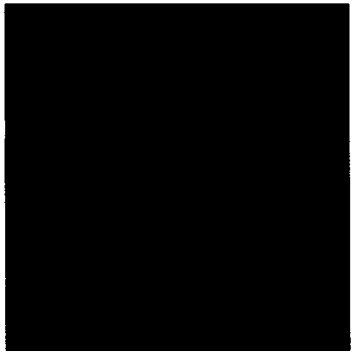


FIGURE 15: Lack of internalization not due to proteosomal degradation of CD127. Preliminary results of IL-7-stimulated $CD8^+$ T cells pre-incubated with lactacystin, a known proteosome inhibitor, did not reveal an increase in cytosolic CD127. (A) CD127 staining on $CD8^+$ T cells treated with IL-7 for 20 minutes. (B) No increase in intracellular CD127 staining in $CD8^+$ T cells pre-treated with lactacystin for 1 hour, followed by IL-7 for 2 minutes.

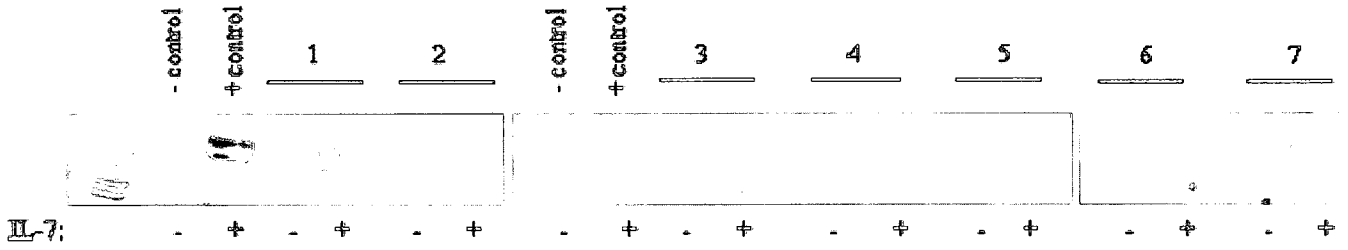

- control
+ control
1
2
- control
+ control
3
4
5
6
7
IL-7:
- + - + - + - + - + - + - + - + - + - +

FIGURE 16: Analysis of soluble CD127 in $CD8^+$ T cell culture supernatants. Western blot analysis of $CD8^+$ T cell culture supernatants was performed to determine if IL-7 induced the release of CD127. Increases in CD127 protein were detected in culture supernatants of $CD8^+$ T cells incubated with IL-7 (1000 pg/ml) for 24 hours (in 5 out of 7 individuals). Equivalent total protein concentrations of culture supernatants collected from untreated WI-26VA4 cells were used as a negative control while IL-7 (10 000 pg/ml)-treated WI-26VA4 cells were included as a positive control.

These results indicate that incubation of $CD8^+$ T cells with IL-7 results in an increase of CD127 in culture supernatants, as could be readily detected in 5 out of 7 samples.

3.2.4 Increased CD127 in the Plasma of HIV^+ Individuals Compared to HIV- Individuals

Since increased plasma IL-7 is associated with HIV-induced lymphopenia [107] and IL-7 induces the shedding of CD127 *in vitro* (FIGURE 16), the plasma of untreated HIV-infected patients was analyzed for the presence of CD127 protein. The CD127-specific Western blot can detect differences in protein concentrations, as demonstrated in a dilution series of a recombinant human CD127-Fc chimera protein with a molecular mass of 80-90 kDa (FIGURE 17A). Analysis of human plasma samples identified three protein bands of approximately 50, 90 and 150 kDa (FIGURE 17B). This finding is similar to previous descriptions in mice in which multiple bands were detected by SDS-PAGE analysis of culture supernatants from cell lines transfected with murine CD127. In the mouse, these protein bands are thought to represent unglycosylated (≈ 49.6 kDa), glycosylated (≈ 68 kDa) and dimeric (≈ 153 kDa) forms of the receptor [108]. Therefore, the multiple protein bands detected here likely represent similar forms of the receptor. Densitometry analysis of the glycosylated CD127 protein bands (≈ 90 kDa) indicated that plasma of untreated HIV^+ individuals (mean CD4 counts 371 cells/ml, mean viral load 98,963 copies/ml) contained significantly more CD127 than HIV^- individuals (FIGURE 17B)

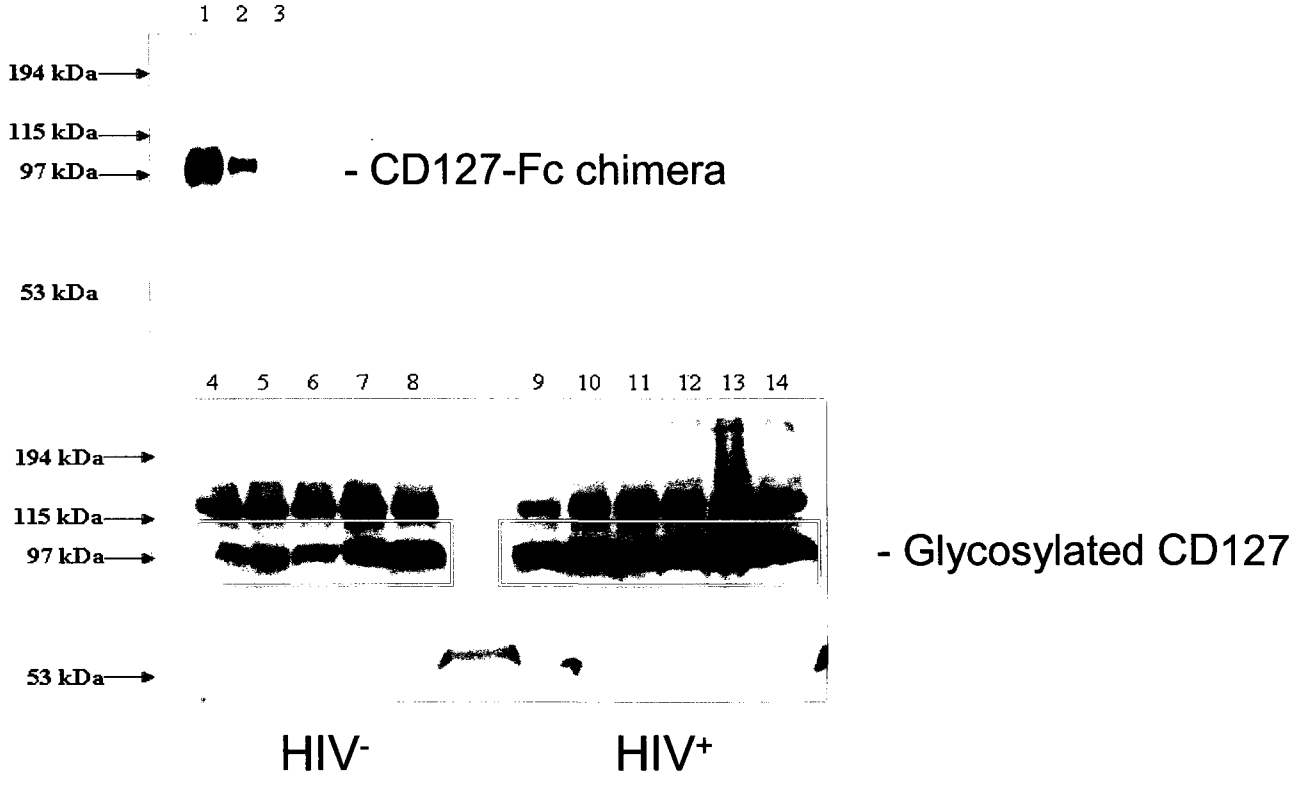

1 2 3
194 kDa
115 kDa
97 kDa
53 kDa
- CD127-Fc chimera
4 5 6 7 8
9 10 11 12 13 14
194 kDa
115 kDa
97 kDa
53 kDa
- Glycosylated CD127
HIV-
HIV+

FIGURE 17: Detection of soluble CD127 in human plasma from healthy and HIV-infected individuals. (A) Detection of different concentrations of human CD127-Fc chimera protein (≈ 80-90 kDa) by Western Blot (lane 1 = 5 ng; lane 2 = 1 ng; lane 3 = 0.1 ng). (B) Analysis of plasma samples from HIV^- (lanes 4-8) and HIV^+ (lanes 9-14) individuals was conducted to determine whether soluble CD127 is present in human plasma and to determine if soluble CD127 is increased in HIV infection *in vivo* Densitometry analysis of the ≈ 90 kDa protein bands, representing the glycosylated CD127 receptor, indicated that HIV^+ individuals have a greater amount of plasma CD127 than HIV^- individuals ($p < 0.001$).

($p = 0.0008$, data not shown). There was no observable difference in the densities of the larger protein band and the smallest protein bands were consistently diffuse and hence densitometry analysis was not performed.

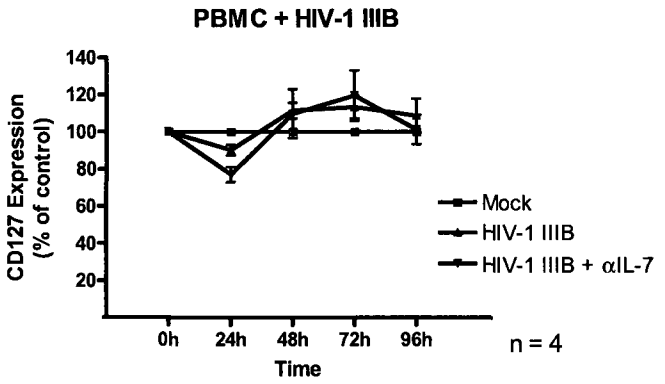
3.3 Determining if *in Vitro* HIV Infection Results in a Down-regulation of CD127 in $CD8^+$ T Cells

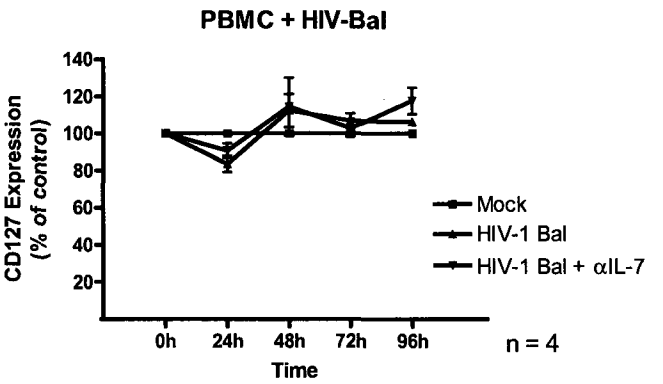
3.3.1 In Vitro HIV-1 Infection Decreases CD127 Surface Expression on $CD8^+$ T Cells in PBMC Cultures

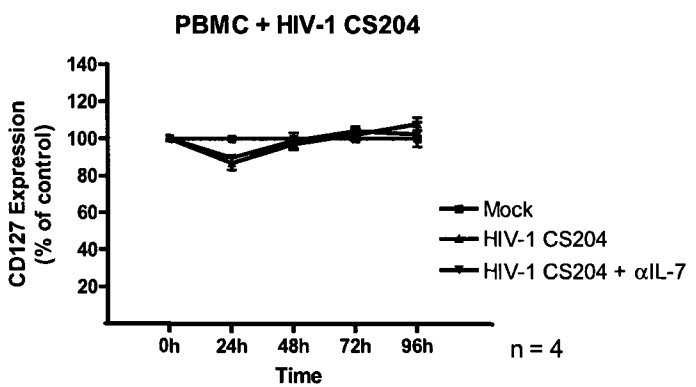
To determine if *in vitro* HIV-1 infection results in a CD127 down-regulation on $CD8^+$ T cells, PBMCs were infected with various strains of HIV-1 and CD127 expression on $CD8^+$ T cells was measured by flow cytometric analysis. HIV-1_{CS204} (FIGURE 18A), HIV-1_{IIIB} (FIGURE 18B) and HIV-1_{Ba-L} (FIGURE 18C) decreased surface CD127 expression on $CD8^+$ T cells in PBMC cultures at 24 hours. The decrease was transient, as CD127 levels increased at 48, 72 and 96 hours. Infection of PBMCs with the non-infective HIV-1 from 8e5 cells, did not affect surface CD127 expression on $CD8^+$ T cells (FIGURE 18D). Polybrene treatment alone did not affect surface CD127 expression on $CD8^+$ T cells (data not shown).

3.3.2 In Vitro HIV-1 Infection Has no Effect on CD127 Surface Expression in Purified $CD8^+$ T Cell Cultures

To determine whether HIV-1 directly affects CD127 expression on $CD8^+$ T cells, purified $CD8^+$ T cells were infected with HIV-1 in parallel to PBMCs.


PBMC + HIV-1 IIIB
CD127 Expression (% of control)
140
120
100
80
60
40
20
0h
24h
48h
72h
96h
Time
Mock
HIV-1 IIIB
HIV-1 IIIB + αIL-7
n = 4


PBMC + HIV-Bal
CD127 Expression (% of control)
140
120
100
80
60
40
20
0h
24h
48h
72h
96h
Time
Mock
HIV-1 Bal
HIV-1 Bal + αIL-7
n = 4


PBMC + HIV-1 CS204
CD127 Expression (% of control)
140
120
100
80
60
40
20
0h
24h
48h
72h
96h
Time
Mock
HIV-1 CS204
HIV-1 CS204 + αIL-7
n = 4

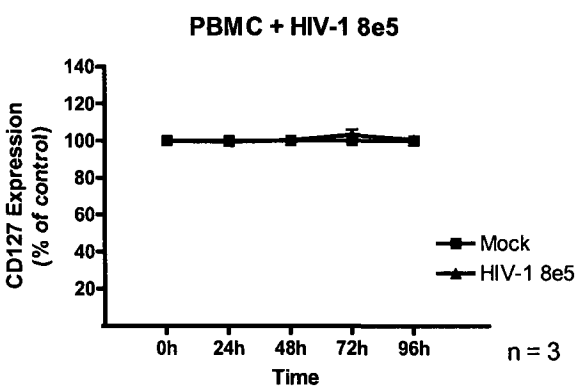

PBMC + HIV-1 8e5
CD127 Expression (% of control)
140
120
100
80
60
40
20
0h
24h
48h
72h
96h
Time
Mock
HIV-1 8e5
n = 3

FIGURE 18: Effect of different strains of HIV-1 on CD127 surface expression on $CD8^+$ T cells in PBMC cultures. To determine if *in vitro* HIV-1 infection can alter CD127 surface expression on $CD8^+$ T cells in PBMC cultures, freshly isolated PBMCs were infected with either (*A*) HIV-1_{IIIB}, (*B*) HIV-1_{BA-L}, (*C*) HIV-1_{CS206}, or (D) supernatants from 8E5 cells, with or without antibodies to IL-7. Infection of PBMCs with HIV-1_{IIIB}, HIV-1_{Ba-L} or HIV-1_{CS206} caused a transient decrease of CD127 on $CD8^+$ T cells. Antibodies IL-7 had no effect on CD127 expression. Supernatants from 8E5 cells had no effect on CD127 expression.

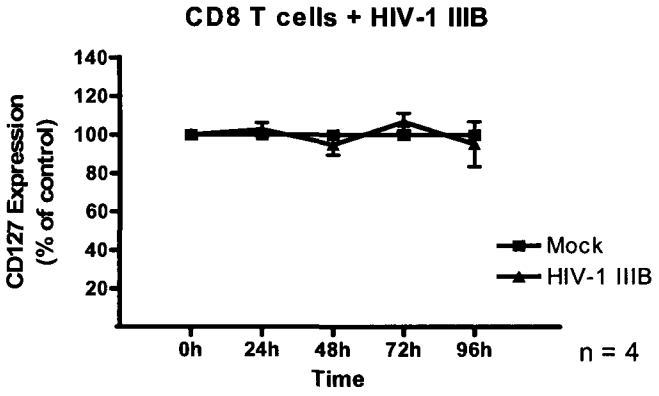
Differing from results in PBMCs, no significant changes in surface CD127 expression were detected on $CD8^+$ T cells following incubation with HIV-1_{IIIB} 9FIGURE 19A), HIV-1_{Ba-L} (FIGURE 19B) or HIV-1_{CS204} (FIGURE 19C). Once again, replication-incompetent HIV from supernatants of 8e5 cells did not affect surface CD127 expression on $CD8^+$ T cells (FIGURE 19D).

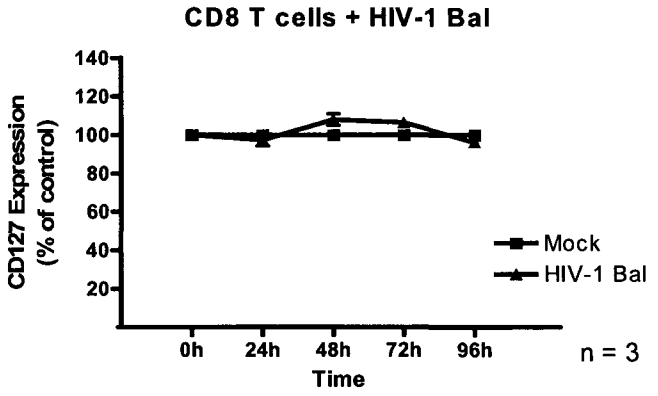
3.3.3 Soluble Factor Generated by HIV-1 Infection Affects CD127 Surface Expression on $CD8^+$ T Cells

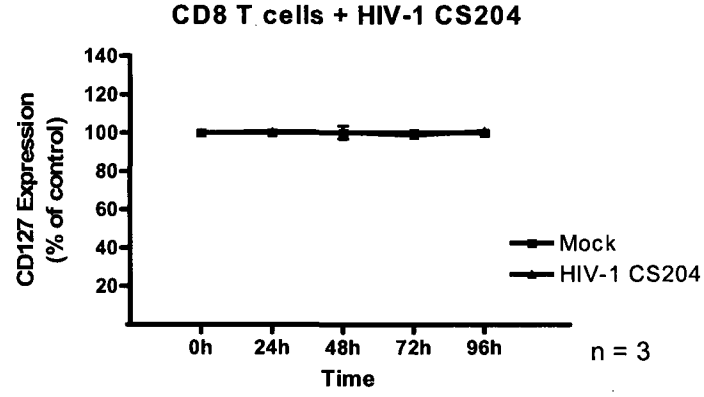
Since *in vitro* HIV-1 infection affects CD127 surface expression on $CD8^+$ T cells within PBMC cultures but not in purified $CD8^+$ T cells alone, the possibility of a factor secreted by PBMCs and affecting CD127 expression was considered. A decrease in surface CD127 expression on $CD8^+$ T cells co-cultured in a transwell system with HIV-infected PBMCs was detected (FIGURE 20). Furthermore, culturing purified $CD8^+$ T cells in supernatants from autologous 24 hour infected PBMCs also resulted in a transient decrease in surface CD127 expression (FIGURE 21), suggesting that a soluble factor(s) may be responsible for down-regulation.

3.3.4 *In vitro* HIV- induced decrease of surface CD127 was not due to increased IL-7 or IL-4 production by PBMCs

As demonstrated in AIM 1, addition of cytokines (IL-7 or IL-4) to PBMCs as well as purified $CD8^+$ T cells significantly down-regulates CD127 surface expression on $CD8^+$ T cells, and led us to investigate the roles of IL-7 and IL-4 in the down-regulation of surface CD127 observed *in vitro* by HIV-1. Analysis of supernatants collected from HIV-1_{CS204} infected PBMCs confirmed that the


CD8 T cells + HIV-1 IIIB
CD127 Expression (% of control)
140
120
100
80
60
40
20
0h
24h
48h
72h
96h
Time
Mock
HIV-1 IIIB
n = 4


CD8 T cells + HIV-1 Bal
CD127 Expression (% of control)
140
120
100
80
60
40
20
0h
24h
48h
72h
96h
Time
Mock
HIV-1 Bal
n = 3


CD8 T cells + HIV-1 CS204
CD127 Expression (% of control)
140
120
100
80
60
40
20
0h
24h
48h
72h
96h
Time
Mock
HIV-1 CS204
n = 3

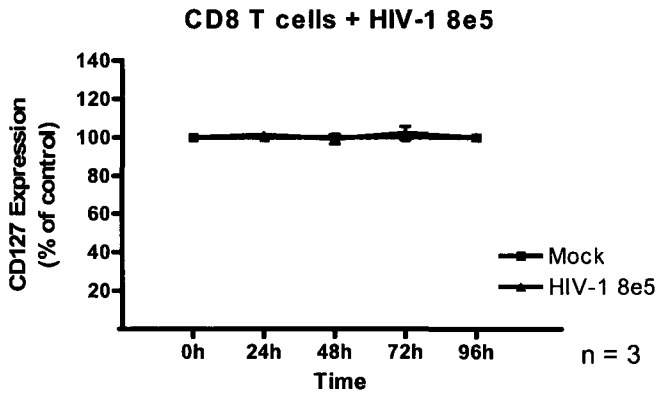

CD8 T cells + HIV-1 8e5
CD127 Expression (% of control)
140
120
100
80
60
40
20
0h
24h
48h
72h
96h
Time
Mock
HIV-1 8e5
n = 3

FIGURE 19: Effect of different strains of HIV-1 on CD127 surface expression on purified $CD8^+$ T cells. To determine if HIV-1 acts directly on $CD8^+$ T cells or via stimulation by some factor/factors secreted by PBMC due to productive infection, freshly isolated $CD8^+$ T cells were cultured with (A) $HIV\text{-}1_{IIIB}$, (B) $HIV\text{-}1_{Ba\text{-}L}$, (C) $HIV\text{-}1_{CS204}$, or (D) supernatants from 8E5 cells. Neither of the HIV strain had an effect on CD127 expression.

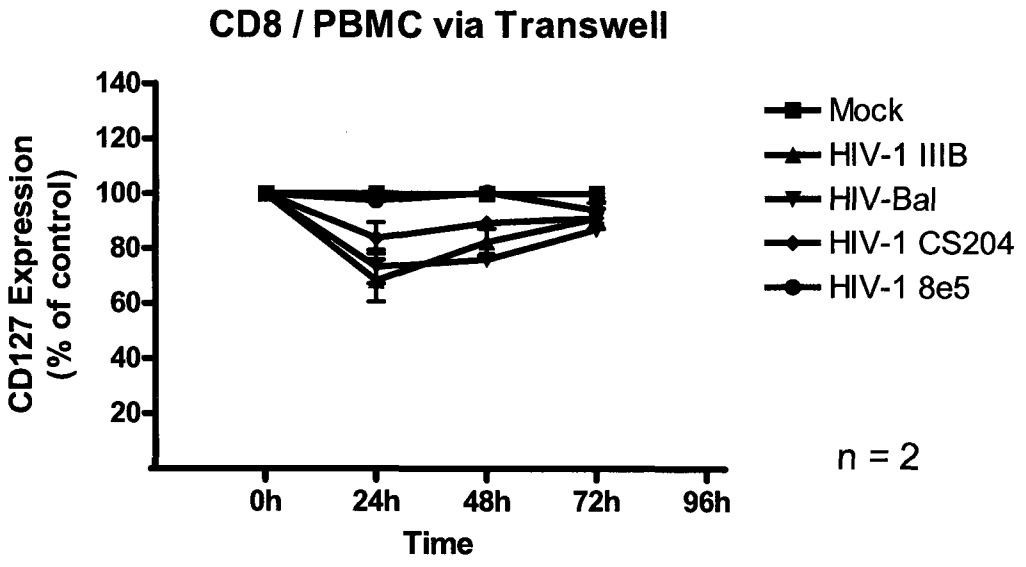

CD8 / PBMC via Transwell
CD127 Expression (% of control)
140
120
100
80
60
40
20
0h
24h
48h
72h
96h
Time
Mock
HIV-1 IIIB
HIV-Bal
HIV-1 CS204
HIV-1 8e5
n = 2

FIGURE 20: Effect of different strains of HIV-1 on CD127 surface expression on purified $CD8^+$ T cells cultured with infected PBMC via a transwell. Purified $CD8^+$ T cells were co-culture via a transwell with freshly isolated PBMCs infected with $HIV\text{-}1_{IIIB}$, $HIV\text{-}1_{BA\text{-}L}$, $HIV\text{-}1_{CS206}$, or supernatants from 8E5 cells. The presence of $HIV\text{-}1_{IIIB}$, $HIV\text{-}1_{BA\text{-}L}$ and $HIV\text{-}1_{CS206}$ infected PBMCs caused a transient decrease of CD127 in purified $CD8^+$ T cells. Supernatants from 8E5 cells had no effect on CD127 expression.

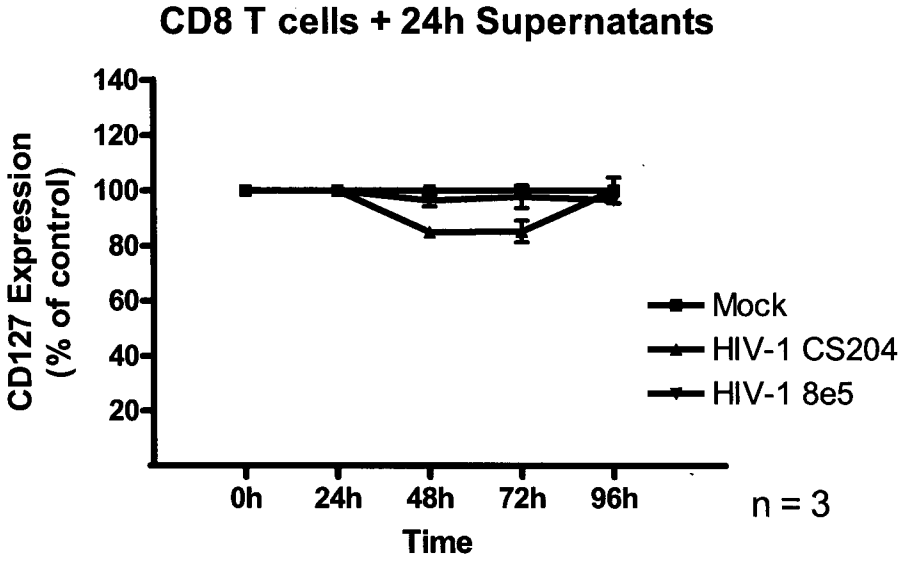

CD8 T cells + 24h Supernatants
CD127 Expression (% of control)
140
120
100
80
60
40
20
0h
24h
48h
72h
96h
Time
Mock
HIV-1 CS204
HIV-1 8e5
n = 3

FIGURE 21: Effect of supernatants from HIV-infected PBMCs on CD127 surface expression on purified $CD8^+$ T cells. Freshly isolated PBMCs were infected with HIV-1_{CS206}, or supernatants from 8E5 cells. After 24 hours, purified $CD8^+$ T cells were culture in the supernatants. CD127 expression on $CD8^+$ T cells was measured by flow cytometry. Supernatants from HIV-1_{CS206} infected PBMCs caused a transient decrease of CD127 on $CD8^+$ T cells. Supernatants from 8E5 cells had no effect on CD127 expression.

decrease in CD127 expression observed on $CD8^+$ T cells in PBMC cultures is not due to an increase in IL-4 (FIGURE 22A) or IL-7 (FIGURE 22B) in supernatants of HIV-1_{CS204} infected PBMCs.

3.3.5 In Vitro HIV-1 Does Not Alter CD127 mRNA Levels

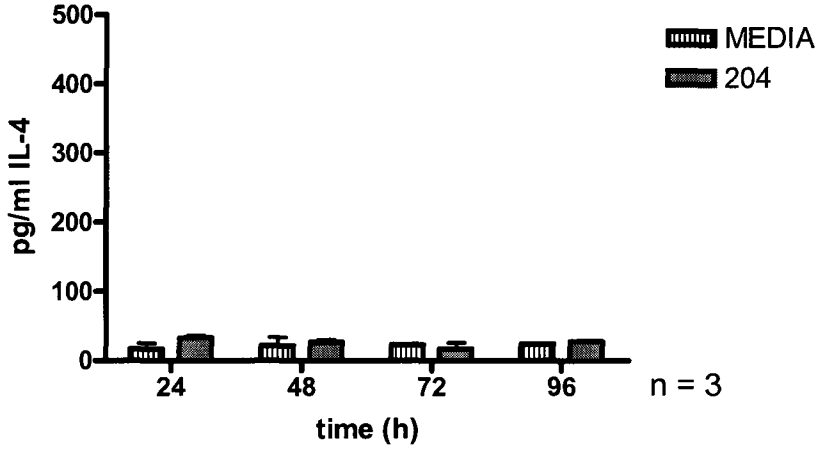
To determine that the observed decrease in surface CD127 was do to a decrease in CD127 mRNA levels, a semi-quantitative PCR method was used to detect expression of mRNA that encoded membrane-bound CD127. HIV-1_{IIIB}, HIV-1_{Bal}, HIV-1_{CS204} and replication-incompetent HIV from supernatants of 8e5 cell line had no effect on CD127 mRNA levels in $CD8^+$ T cells in PBMC cultures at 24 hours (FIGURE 23).

3.4 Determining if the Functional Capacity of CD127 is Altered in HIV Infection

3.4.1 Patient Subjects and Study Design

The functional capacity of CD127 was studied in a cross-sectional study. This study included 6 healthy volunteers and 18 HIV-infected individuals at distinct stages of disease progression. This included 13 untreated HIV-infected individuals and 5 treated HIV-infected individuals. Untreated patients were naïve to antiretroviral therapy or off therapy for at least a year. Treated patients have been on effective antiretroviral therapy for more than a year, with CD4 counts > 400 cells/mm^3 and undetectable viral load (VL) (FIGURE 24).

A


500
400
300
200
100
0
pg/ml IL-4
24
48
72
96
time (h)
MEDIA
204
n = 3

B

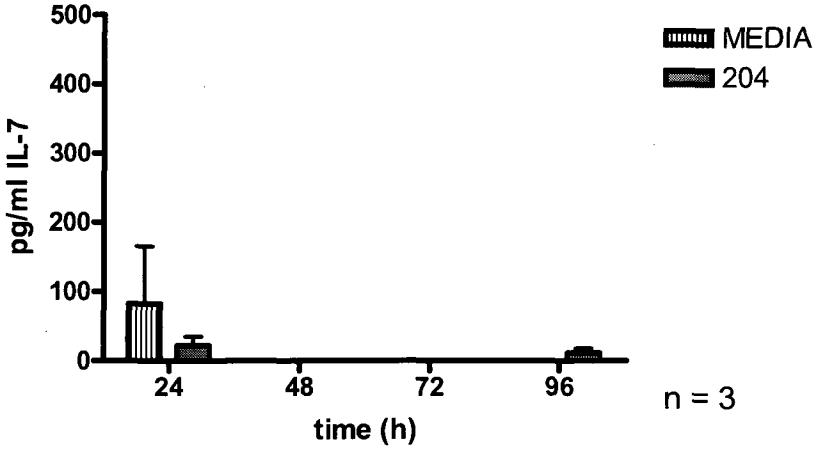

500
400
300
200
100
0
pg/ml IL-7
24
48
72
96
time (h)
MEDIA
204
n = 3

FIGURE 22: **In vitro HIV-induced decrease in surface CD127 expression on $CD8^+$ T cells is not due to increased IL-4 or IL-7 production.** Analysis of supernatants collected from HIV-1_{CS204} infected PBMCs showed no significant increase in soluble IL-4 (A) or IL-7 (B).

Mock
HIV-1 IIIB
HIV- Ba-L
HIV-1 CS204
HIV-8e5
CD127
β-actin

FIGURE 23: Effect of different strains of HIV-1 on CD127 gene expression in $CD8^+$ T cells. The *in vitro* effect of different strains of HIV-1 on CD127 gene expression was studied to further evaluate the mechanism of CD127 down-regulation on $CD8^+$ T cells. Addition of $HIV\text{-}1_{IIIB}$, $HIV\text{-}1_{Ba\text{-}L}$, $HIV\text{-}1_{CS204}$ or supernatants from 8E5 cells for 24 hours did not alter the amount of mRNA encoding membrane-bound CD127 expressed by $CD8^+$ T cells as determined by semi-quantitative PCR. The molecular weight marker is shown on the left. (one representation of three)

PATIENT	D.O.B.	CD4 COUNT	CD4%	VL	TX STATUS
AK	26/06/81	611	41.9	74 711	OFF IN 2006
NNI	10/10/73	579	19.4	6 030	NEVER
BP	19/02/74	151	14.8	365 301	OFF 1.5 YRS
SS	12/08/48	565	25.8	44 133	OFF IN 2004
DEB	23/12/50	327	19.0	20 865	OFF IN 2000
PP	30/12/81	585	21.2	4 410	NEVER
SBC	21/03/90	282		10 026	OFF 2-3 YRS
MF	16/05/70	286	22.1		NEVER
JRM	18/06/83	454	23.9	76 693	NEVER
KLH	23/09/62	170	28.2	65 416	NEVER
IT	12/12/56	415	8.7	22 910	NEVER
FZT	01/01/76	126	17.3	303 487	NEVER
CL	10/05/80	592	38.8	12 549	NEVER
JAS	18/11/75	438	31.2	58 405	NEVER
JW	11/03/72	282	16.6	14 337	NEVER
AN	15/10/64	532	25.3	< 50	NEVER
CR	05/09/63	575	47.1	15 385	NEVER
LSAM	26/11/68	171	10.3	178 966	NEVER
JP	29/04/71	120	21.0	4 529	NEVER
DLB	11/12/64	354	29.6	1 945	OFF IN 2003
WR	01/06/78	237	9.8	543	NEVER
GWB	01/01/70	198	14.6	67 516	NEVER
TG	07/05/71	902	42.7		TX
OM	21/06/50	1143	32.8	< 50	TX
TT	10/11/59	358	22.1	< 50	TX
GL	18/07/62	887	44.2	< 50	TX
VF	03/10/43	523		< 50	TX

FIGURE 24: CD4 count, VL and therapy status of HIV-infected patients. This cross-sectional study included 18 HIV-infected individuals at distinct stages of disease progression. This included 13 untreated HIV-infected individuals and 5 treated HIV-infected individuals. Untreated patients were naïve to antiretroviral therapy or off therapy for at least a year. Treated patients have been on effective antiretroviral therapy for more than a year, with CD4 counts > 400 cells/mm^3 and undetectable VL (VL<50 copies/ml).

3.4.2 Phenotypic Analysis of Patient PBMCs

PBMCs from participants were analyzed for expression levels of the cell surface markers CD8 and CD127. As previously seen in our laboratory and by others [83], CD127 expression levels on $CD8^+$ T cell were decreased in untreated HIV-infected individuals when compared to healthy controls. Patients on effective antiretroviral therapy had slightly increased levels of CD127 on $CD8^+$ T cells compared to untreated patients (FIGURE 25).

3.4.3 Decreased IL-7-Induced P-STAT5 Activation in HIV-Infected Individuals

IL-7/IL-7R signalling plays an essential role in the proper functioning and maintenance of T cell, as well as their proliferation and differentiation. Cytokine-cytokine receptor interaction has been shown to activate various signalling pathways, one of which is being the JAK/STAT pathway. Therefore, IL-7-induced STAT5 phosphorylation (P-STAT5) in $CD8^+CD127^+$ T cells from healthy and untreated HIV-infected individuals was investigated. Mean channel fluorescence (MFI) as well as percent expression were used to assess the level of STAT5 phosphorylation. A significant decrease, both in percent expression (FIGURE 24B, left panel) and MFI (FIGURE 26A and 26B, right panel), in IL-7 induced STAT5 phosphorylation were detected in untreated HIV-infected individuals compared to healthy controls ($p=0.0013$ for % expression and $p<0.001$ for MFI).

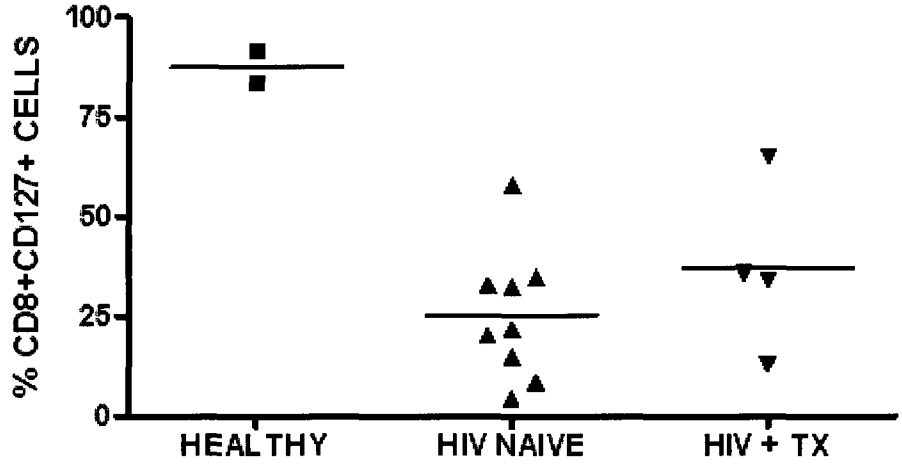

% CD8+CD127+ CELLS
100
75
50
25
0
HEALTHY
HIV NAIVE
HIV + TX

FIGURE 25: CD127 expression levels on $CD8^+$ T cells. CD127 expression levels on $CD8^+$ T cell are decreased in untreated HIV-infected individuals when compared to healthy controls. Patients on effective antiretroviral therapy had increased levels of CD127 on $CD8^+$ T cells compared to untreated patients.

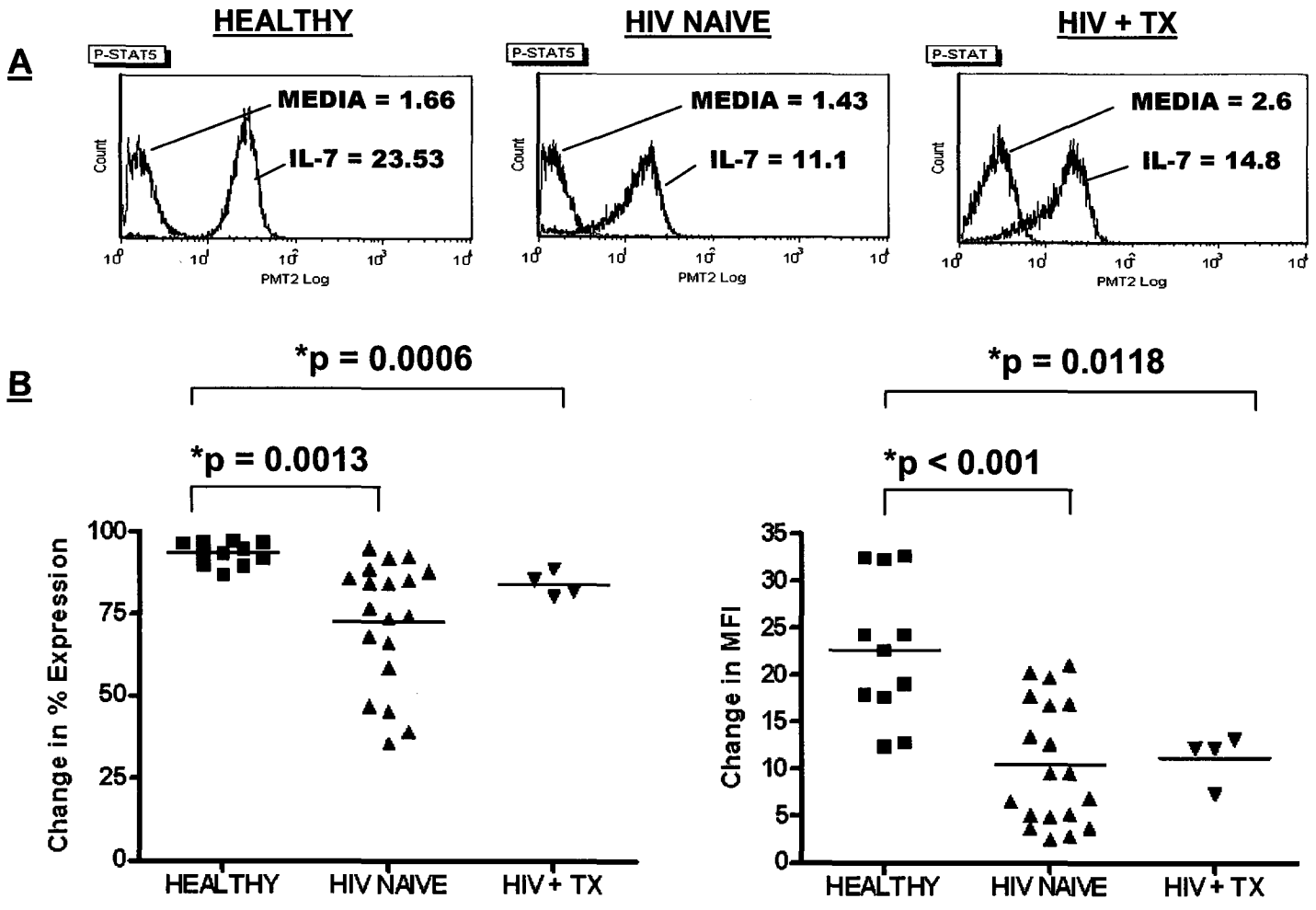

A
HEALTHY
HIV NAIVE
HIV + TX
P-STAT5
P-STAT5
P-STAT
MEDIA = 1.66
IL-7 = 23.53
MEDIA = 1.43
IL-7 = 11.1
MEDIA = 2.6
IL-7 = 14.8
Count
PMT2 Log
B
*p = 0.0006
*p = 0.0013
*p = 0.0118
*p < 0.001
Change in % Expression
Change in MFI
HEALTHY
HIV NAIVE
HIV + TX

FIGURE 26: HIV-infected individuals have decreased IL-7-induced phophorylation of STAT5. IL-7-induced STAT5 phosphorylation (P-STAT5) in $CD8^+CD127^+$ T cells from healthy, untreated, and treated HIV-infected individuals was investigated. (A) Overlaid flow cytometric histograms demonstrating a decrease in IL-7 induced P-STAT5 MFI in the untreated (HIV naïve) and treated (HIV^+TX) patients compared to healthy controls (11.1 and 14.8 vs. 23.53). (B) A significant decrease, both in percent expression (left panel) and MFI (right panel), in IL-7 induced STAT5 phosphorylation were detected in untreated HIV-infected individuals compared to healthy controls ($p=0.0013$ for % expression and $p<0.001$ for MFI by t-test). Patients on antiretroviral therapy also had decreased STAT5 phosphorylation compared to healthy controls ($p=0.0006$ for % and $p=0.0118$ for MFI by t-test).

Patients on antiretroviral therapy also had decreased STAT5 phosphorylation compared to healthy controls ($p=0.0006$ for % and $p=0.0118$ for MFI). No significant correlation have been detected between CD127 expression levels and phosphorylated STAT5 levels (FIGURE 27A) as well as between CD4 counts and phosphorylated STAT5 levels (FIGURE 27B)

3.4.4 No Change in IL-7-Induced Bcl-2 Production Between Healthy and HIV-Infected Individuals

It has been well described that IL-7 is required for homeostatic survival of T cells and that this survival may be attributed to the production of anti-apoptotic molecules such as Bcl-2, which offset cell death signals. Numerous groups have shown that IL-7-induced Bcl-2 production plays a critical role in lymphoid development and regulation of immune responses [109]. Therefore, the IL-7-induced expression of Bcl-2 in isolated $CD8^+CD127^+$ from healthy and untreated HIV-infected individuals was investigated. Mean channel fluorescence (MFI) as well as percent expression were used to assess the level of Bcl-2 expression. In these experiments, there was no statistically significant difference in the amount IL-7-induced Bcl-2 expressed in $CD8^+CD127^+$ T cells from healthy, untreated or treated HIV-infected individuals (FIGURE 28). Treated HIV-infected individuals did however demonstrated higher Bcl-2 expression levels in their media control compared to treated HIV-infected and healthy individuals (FIGURE 29).

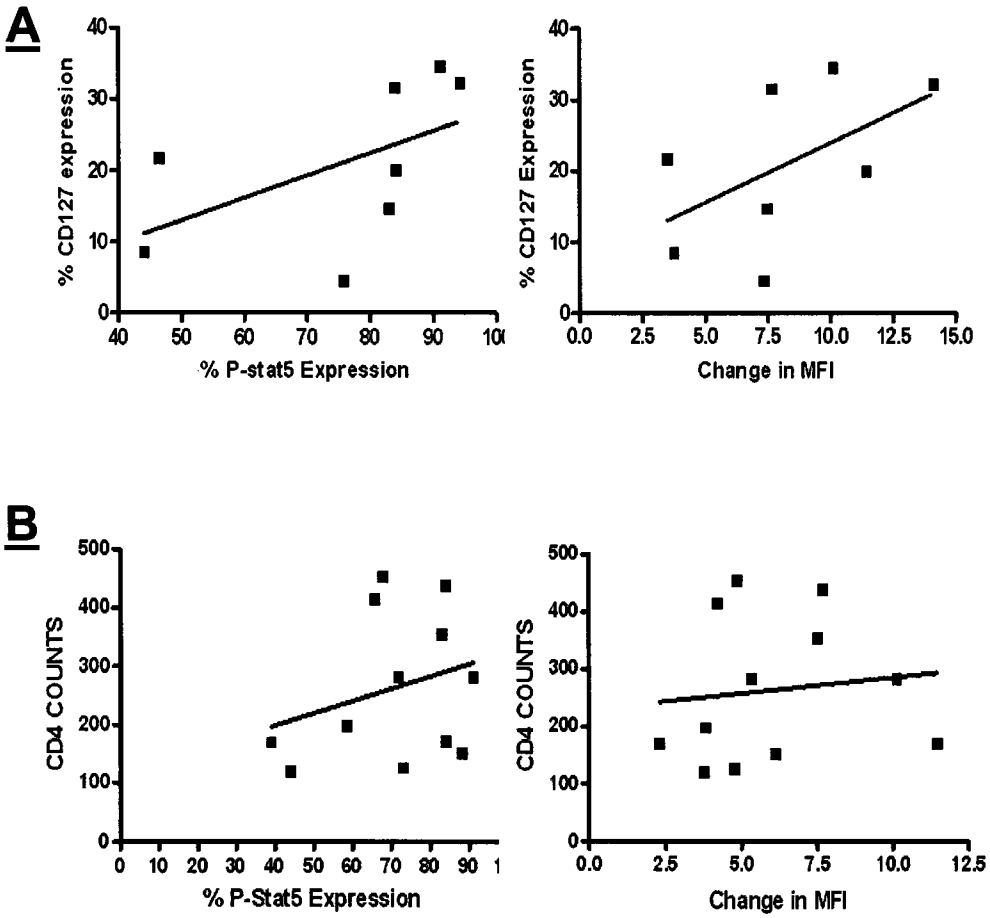

A
% CD127 expression
% P-stat5 Expression
% CD127 Expression
Change in MFI
B
CD4 COUNTS
% P-Stat5 Expression
CD4 COUNTS
Change in MFI

FIGURE 27: **Correlations between CD127 expression levels or CD4 counts and P-STAT.** No significant correlations have been demonstrated between untreated HIV-infected individuals' CD127 expression on $CD8^+$ T cells vs. IL-7-induced P-STAT5 (A) or CD4 counts vs. IL-7-induced P-STAT 5.

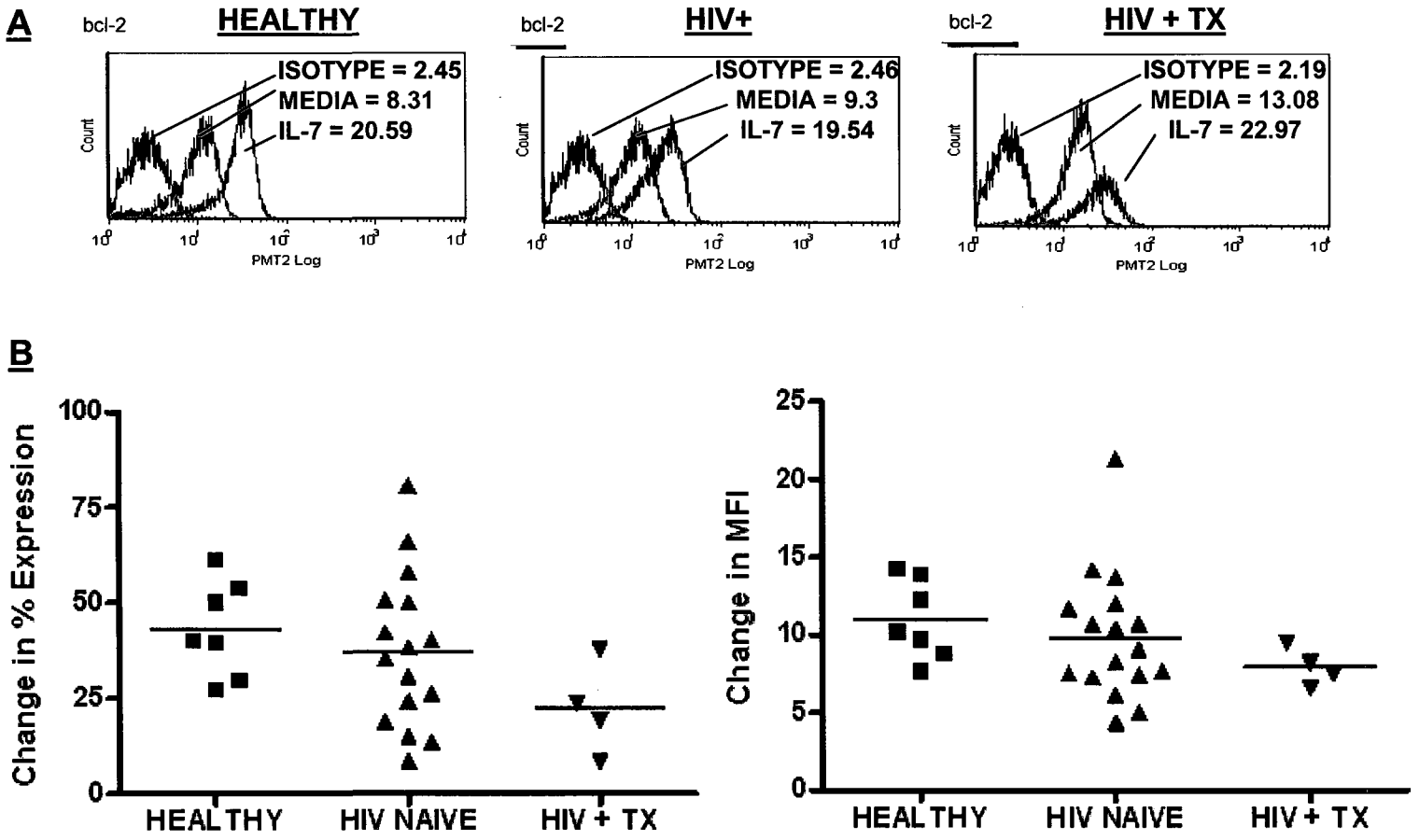

A
bcl-2
HEALTHY
ISOTYPE = 2.45
MEDIA = 8.31
IL-7 = 20.59
Count
PMT2 Log
bcl-2
HIV+
ISOTYPE = 2.46
MEDIA = 9.3
IL-7 = 19.54
Count
PMT2 Log
bcl-2
HIV + TX
ISOTYPE = 2.19
MEDIA = 13.08
IL-7 = 22.97
Count
PMT2 Log
B
Change in % Expression
100
75
50
25
0
HEALTHY
HIV NAIVE
HIV + TX
Change in MFI
25
20
15
10
5
0
HEALTHY
HIV NAIVE
HIV + TX

FIGURE 28: Interleukin-7-induced Bcl-2 expression levels in healthy, untreated and treated HIV-infected individuals. IL-7-induced Bcl-2 expression in $CD8^{+}CD127^{+}$ T cells from healthy, untreated and treated HIV-infected individuals was investigated. (A) Overlaid flow cytometric histograms demonstrating no change in IL-7 induced Bcl-2 MFI in the untreated (HIV naïve) and treated ($HIV^{+}TX$) patients compared to healthy controls (19.54 and 22.9 vs. 20.59). (B) No significant differences in percent expression (left panel) and MFI (right panel), in IL-7 induced Bcl-2 were detected between healthy, untreated and treated HIV-infected individuals.

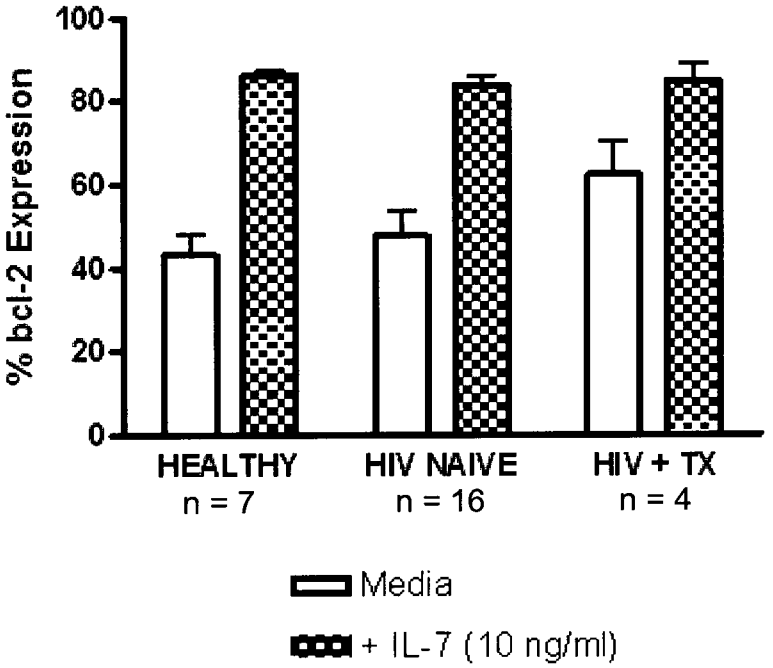

% bcl-2 Expression
100
80
60
40
20
0
HEALTHY
n = 7
HIV NAIVE
n = 16
HIV + TX
n = 4
Media
+ IL-7 (10 ng/ml)

FIGURE 29: HIV-infected individuals on therapy have increased baseline Bcl-2 expression. IL-7-induced Bcl-2 expression in $CD8^+CD127^+$ T cells from healthy, untreated and treated HIV-infected individuals was investigated. Following 48hours in culture with media alone or IL-7, the media treated $CD8^+CD127^+$ T cells from treated HIV-infected individuals have higher Bcl-2 expression compared to healthy or untreated HIV-infected individuals.

3.4.5 No Change in IL-7-Induced CD107a/b Expression Between Healthy and HIV-Infected Individuals

One mechanism by which $CD8^+$ T cells mediate cytolytic activity is by the direct release of lytic granules containing perforin and granzyme and hence the expression of LAMPs such as CD107a (LAMP-1) and CD107b (LAMP-2) was evaluated. Although not normally expressed on the surface of $CD8^+$ T cells, expression of CD107a and CD107b have been observed following T cell activation [110]. Therefore, we measured the effect of IL-7 on the expression of CD107a and b on $CD8^+$ $CD127^+$ T cells in healthy and untreated HIV-infected individuals. Addition of IL-7 did not alter CD107a and CD107b surface expression on $CD8^+$ $CD127^+$ T cells in both healthy and HIV infected individuals, and no significant change in IL-7-induced expression of CD107a or CD107b was seen between healthy and untreated HIV-infected individuals (FIGURE 30).

3.4.6 Impaired IL-7-Induced $CD8^+CD127^+$ T Cells Proliferation in Untreated HIV-Infected Individuals

It has been well described that IL-7 promotes the survival and proliferation of $CD8^+$ T cells; therefore, we studied the effects of IL-7 on cell division of mitogen-stimulated $CD8^+CD127^+$ T cells. Incubation of $CD8^+CD127^+$ T cells with IL-7 alone did not result in an appreciable number of cells undergoing multiple cell divisions, i.e. % $CFSE^{lo}$, therefore a low dose (sub-maximal) dose of PHA (0.5 ug/ml) was used to stimulate T cells *in vitro* in order to study the effects of IL-7 (FIGURE 31).

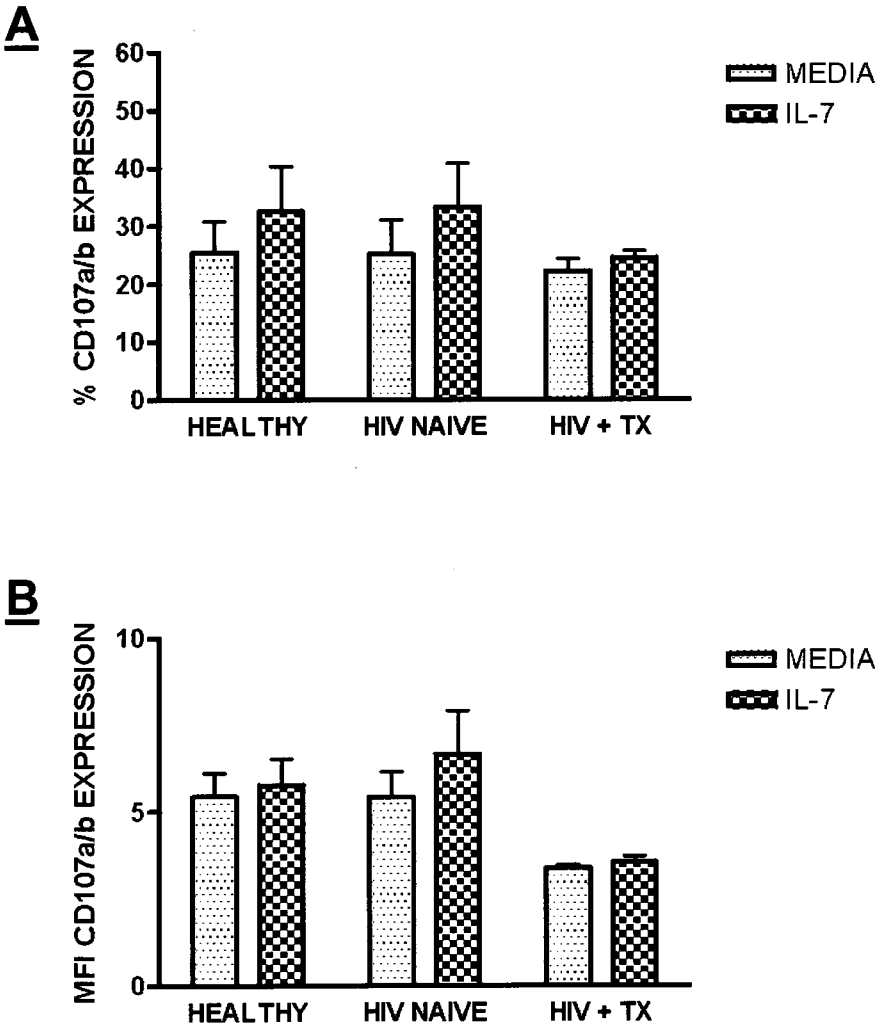
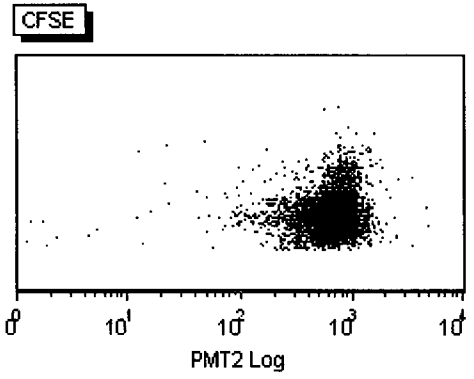
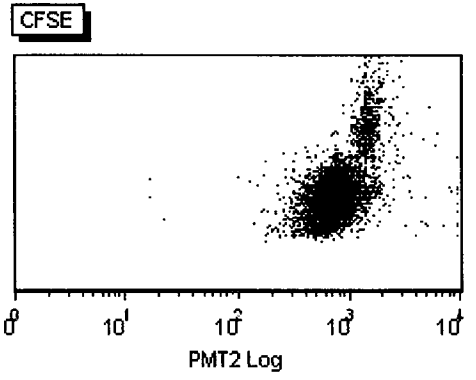

A
% CD107a/b EXPRESSION
60
50
40
30
20
10
0
HEALTHY
HIV NAIVE
HIV + TX
MEDIA
IL-7
B
MFI CD107a/b EXPRESSION
10
5
0
HEALTHY
HIV NAIVE
HIV + TX
MEDIA
IL-7

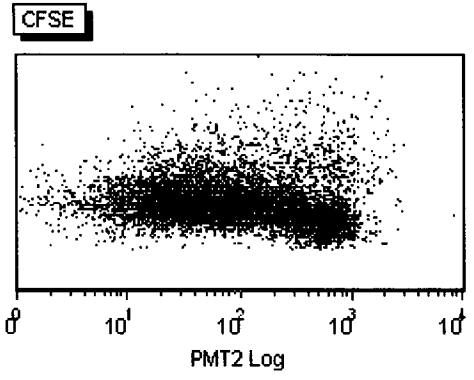
FIGURE 30: IL-7 induced expression of CD107a and CD107b in healthy, untreated and treated HIV-infected individuals. The effect of IL-7 on the expression of CD107a and b on $CD8^+$ $CD127^+$ T cells in healthy, untreated and treated HIV-infected individuals was studied. No significant change in IL-7-induced expression of CD107a or CD107b was seen between healthy and untreated HIV-infected individuals

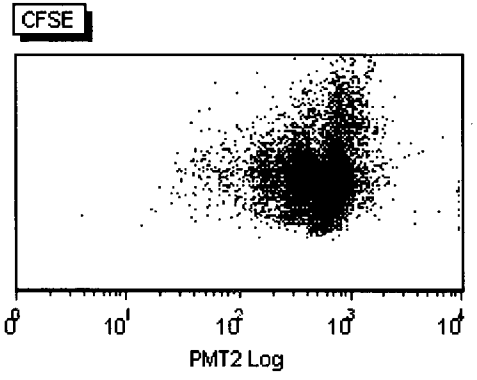
PHA
0.5 ug/ml





PHA
0.5 ug/ml
+
IL-7
10 ng/ml



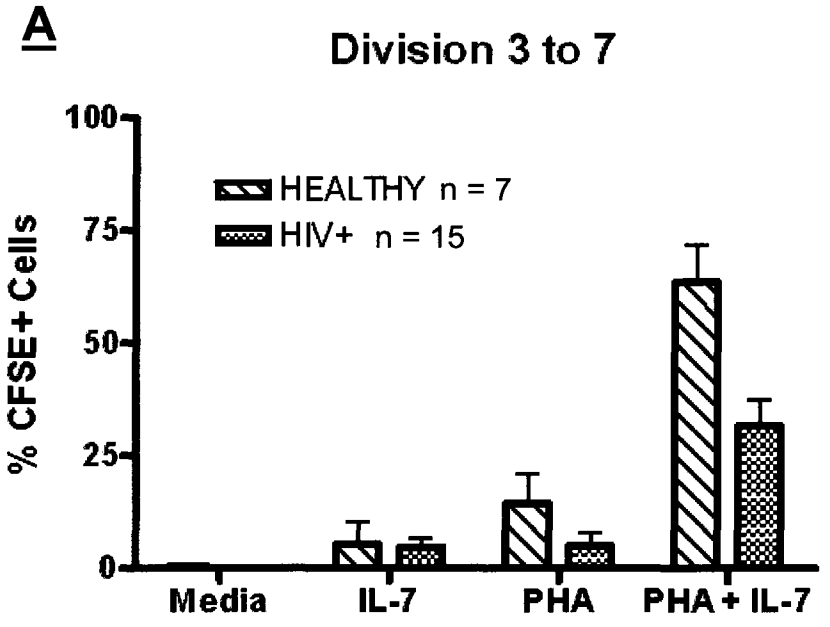


HEALTHY

HIV+

FIGURE 31: Sub-maximal dose of PHA is required to study the effects of IL-7 on T cell proliferation. Flow cytometric histograms demonstrating differences in IL-7-induced proliferation between healthy and HIV-infected individuals. IL-7 alone did not result in an appreciable number of cells undergoing multiple cell divisions. Incubation of $CD8^{+}CD127^{+}$ T cells with a low dose (sub-maximal) of PHA (0.5 ug/ml) was used to stimulate T cells *in vitro* in order to study the effects of IL-7. Sub-maximal dose of PHA in healthy and untreated HIV-infected individuals did not result cells undergoing multiple cell divisions, however, sub-maximal dose of PHA in combination with IL-7 resulted in an appreciable number of cells undergoing multiple cell divisions, allowing us to study the effects of IL-7.

Addition of IL-7 significantly increased the proportion of $CFSE^{lo}$ PHA- stimulated $CD8^{+}CD127^{+}$ T cells compared to cells cultured with PHA alone. $CD8^{+}CD127^{+}$ T cells from healthy controls divided significantly more compared to untreated HIV-infected individuals in response to IL-7 (FIGURE 32).


A
Division 3 to 7
% CFSE+ Cells
100
75
50
25
0
HEALTHY n = 7
HIV+ n = 15
Media
IL-7
PHA
PHA + IL-7

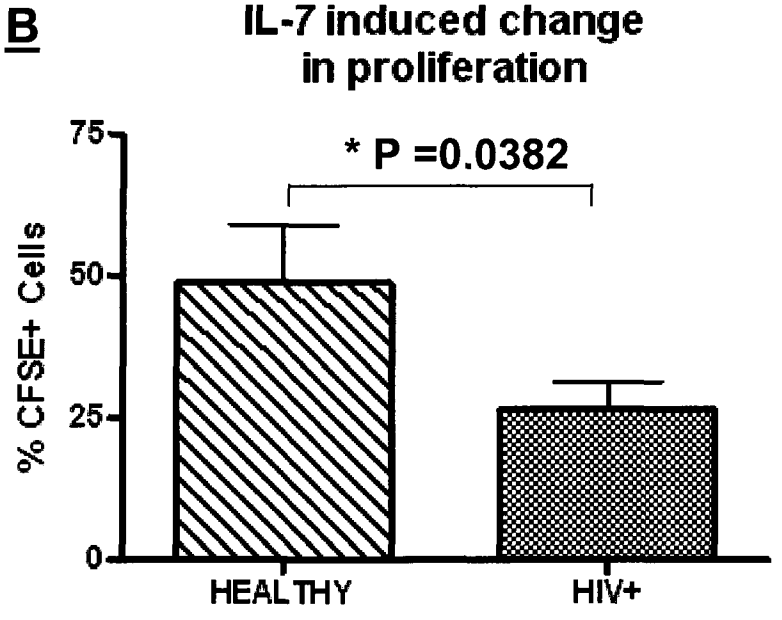

B
IL-7 induced change in proliferation
* P =0.0382
% CFSE+ Cells
75
50
25
0
HEALTHY
HIV+

FIGURE 32: IL-7-induced proliferation is impaired in HIV-infected individuals. (A) Incubation of CD8+CD127+ T cells with a low dose (sub-maximal) of PHA (0.5 ug/ml) was used to stimulate T cells *in vitro* in order to study the effects of IL-7. Sub-maximal dose of PHA resulted in a limited number of cells undergoing multiple divisions. The addition of IL-7 resulted in an appreciable number of cells undergoing multiple divisions, allowing us to study the effects of IL-7. (B) CD8+CD127+ T cells from healthy controls divided significantly more compared to untreated HIV-infected individuals in response to IL-7. In untreated HIV-infected individuals, the addition of IL-7 resulted in a smaller increase in the absolute number of CFSE-labelled cells undergoing divisions 3 to 7 compared to healthy controls. ($p=0.038$ by t-test).

4 DISCUSSION

4.1 Determining which host or viral factor(s) may be responsible for the down-regulation of CD127 on $CD8^+$ T cells in HIV infection, and the mechanism thereof

Reduced CD127 expression on $CD8^+$ T cells of HIV-infected patients with uncontrolled viral replication may contribute to the observed decline in CTL activity and provides a rationale for investigating potential host and viral factors that may alter CD127 expression and the mechanism(s) thereof. Although HIV-specific CTLs persist in the circulation during HIV infection, they are unable to control viral replication [21] and may contribute to ensuing susceptibility to opportunistic infections and malignancies. Down-regulation of T cell-associated CD3-ζ chain [17], reduced perforin [19] and interferon-γ expression [20], maturation arrest of $CD8^+$ effectors T cells [21] and increased PD-1 expression [111-113] have been identified as potential causes for decreased CTL activity during HIV infection. However, the extent to which each of these contributes to impaired CTL activity and the mechanisms by which each of these occurs remains unclear.

The survival and proliferation of lymphoid progenitors is dependent on IL-7R signalling, however the control of receptor expression remains poorly understood. Regulation of murine CD127 gene expression in hematopoeitic progenitors is dependent on the transcription factor PU.1 binding to a GGAA motif, affecting primarily pro-B cell development [39]. In contrast, murine T cells

do not express PU.1 but rather depend on a GA binding protein binding to the same GGAA motif to regulate CD127 expression [40]. Therefore, regulation of CD127 differs in distinct lymphoid lineages in part by the differential recruitment of factors to the same GGAA motif. In addition to reports on factors downregulating CD127 in mice, glucocorticoids such as dexamethasone, have been shown to up regulate human and mouse CD127 gene and protein expression [41, 114], suggesting that CD127 transcription is inducible.

Patients with HIV infection have increased circulating IL-7 concentrations, and this may be a compensatory response to the observed T cell depletion [107, 115]. Paradoxically, IL-7 may down-regulate the expression of its own receptor, as occurs with other cytokines including IL-15 [116] whose receptor shares the IL-2 receptor γ chain with CD127. Cytokine-mediated down-regulation of CD127 expression has been reported in IL-2-treated PBMCs from HIV^- [43] and in HIV^+ individuals receiving IL-2 therapy [117, 118].

4.1.1 Role of Host Factors in the Down-regulation of CD127 on $CD8^+$ T cells

In vivo and *in vitro* studies have shown that HIV infection induces the production of pro-inflammatory cytokines such as IL-1-β, IL-6 and TNF-α. In some cases, direct correlations between the concentration of pro-inflammatory molecules and viral replication have been observed [119, 120]. Similarly, immunoregulatory cytokines such as IL-10 and TGF-β have also been shown to play a role in HIV infection by impairing T cell function and suppressing both HIV-specific and unrelated immune responses [121-123]. However, none of these molecules studied had an effect on CD127 expression on $CD8^+$ T cells. Patients

with HIV disease have increased circulating IL-7 concentrations (26), and this may be a compensatory response to the down-regulation of CD127. Conversely, increases in IL-7 may down-regulate the expression of its own receptor as occurs with other cytokines including IL-15 whose receptor shares the common gamma chain with CD127 [116].

In the present study, IL-7 and IL-4 decreased CD127 surface expression on $CD8^+$ T cells in both PBMCs and isolated $CD8^+$ T cells *in vitro*, which may reflect the observed decrease in CD127 expression in HIV-infected individuals [83]. These results are consistent with recent observations by Colle *et al.* [84] and Crawley *et al.* (in preparation). Concentrations of IL-7 in the plasma of HIV-infected individuals have been shown to be elevated up to 70 pg/ml [23, 107, 115, 124, 125] in the range of those which caused a transient decrease in the expression of CD127 in the experiments here (FIGURE 1 and 3) and suggesting that these observed changes may be physiologically relevant. Moreover, these changes were most evident within the naïve $CD8^+$ T cell population ($CD45RA^+$). The regulation of CD127 early in the T cell life cycle is thought to be associated with the competition for sometimes limiting sources of IL-7 in relation to the numbers of circulating T-cells, particularly naïve T cells which compete with one another for the receipt of IL-7 survival signals [45]. Concentrations of IL-4 in the plasma of HIV-infected individuals have been shown to be elevated [107, 126-128] at levels which caused a decrease in the expression of CD127 over time and suggest these findings are also physiologically relevant. The ability of $CD8^+$ T cells to suppress HIV replication has been reported to be decreased by the

presence of IL-4 [129]. Since IL-4 affects CD127 expression on $CD8^+$ T cells, the loss in $CD8^+$ CTL anti-HIV activity with disease progression may be due in part to the decreased CD127 expression on $CD8^+$ T cells. Evaluating the role of IL-4 induced down-regulation of CD127 and the anti-HIV activity of CTL should be further studied.

Since IL-7 and IL-4 decreased cell surface CD127 expression without altering expression of mRNA encoding membrane-bound CD127, it is possible that CD127 expression may be regulated post-transcriptionally. The transient nature of CD127 down-regulation on $CD8^+$ T cells treated with up to 1000 pg/ml of IL-7 is consistent with the effects of IL-7 on CD127 surface expression on $CD4^+$ T cells from healthy human donors [130] in the absence of changes in mRNA encoding membrane-bound CD127 (personal communication with S. Sasson and A. Kelleher). In contrast, a recent report suggests that human $CD4^+$ T cells treated with IL-7 down-regulate both CD127 surface expression and production of total CD127 mRNA (i.e. transcripts that would either encode membrane bound or secreted receptor) [131], albeit only after 3-5 days of culture. In mice, IL-7-treated $CD8^+$ T cells down-regulate CD127 transcription [45], however that report did not specify whether the measured transcripts included mRNA encoding membrane bound CD127. These differences in CD127 gene regulation by IL-7 may be due to different experimental systems or inter-species variation. An example where such a difference exists is in the treatment of HIV-infected individuals where IL-2 does not decrease surface CD127 expression on $CD4^+$ or $CD8^+$ T cells [118], while similar treatment of mice significantly

decreases the expression of CD127 protein and mRNA in lymph node and splenic T cells [43, 45]. Human-murine species differences in CD127 and transcription factor mRNA expression have become increasingly apparent. Decreased human CD127 mRNA expression was associated with decreased GA binding protein expression [132], while increased CD127 mRNA expression was associated with increased expression of growth factor independence-1 which is known to suppress murine CD127. Although a splice variant of CD127 encodes a secreted form of the receptor is known to exist [108], the effects of IL-7 and other IL-2Rγ cytokines on the expression of mRNA encoding secreted CD127 have not been studied and is the focus of future research.

A possible mechanism for surface receptor down-regulation is receptor internalization, a common feature of many cytokine receptors. In the case of CD127, IL-7 (10 – 1000 pg/ml) transiently decreases surface receptor expression on $CD8^+$ T cells without affecting mRNA expression. Therefore, we investigated the possibility of receptor internalization. Recent evidence suggests that IL-7-induced phosphorylation of tyrosine residues in the intracellular domain of CD127 may lead to an association with clathrin, a protein known to be required for receptor internalization [133]. Moreover, the amino acid sequence of CD127 contains 2 tyrosine signal sequences (401-404 YQDL and 449-452 YVTM) in the intracellular domain that are thought to be part of receptor internalization motifs targeting transmembrane proteins to endocytic or secretory pathways, as reported in the IL-2α, β and γ receptors [105, 134]. Although cytoplasmic CD127 was detected (FIGURE 13B-D) within $CD8^+$ T cells, IL-7 does seem to enhance

receptor internalization. It was also noted that the amount of cytoplasmic CD127 in $CD8^+$ T cells was significantly less than IL-2Rγ (FIGURE 5A), suggesting that spontaneous receptor recycling or cytokine-mediated receptor internalization may not be a primary mechanism for downregulating cell surface expression. In support of these findings, it has been suggested by others that CD127 is not significantly internalized in HIV infection, despite the correlation between increased IL-7 production and decreased surface CD127 expression [130].

Since IL-7 did not induce an appreciable increase in cytoplasmic CD127, suggesting that the down-regulation of CD127 was not a result of receptor internalization, we investigated whether CD127 is shed from T-cells. We were able to detect the release of CD127 from IL-7 stimulated $CD8^+$ T cells in 5 of 7 individuals as determined by Western blot. Since Western blot analysis is typically capable of detecting down to a minimum of approximately 0.1 ng/ml of protein, the fact that soluble CD127 was not observed in 2 individuals likely reflects the limited sensitivity of the assay used. In addition, further work in our laboratory by Dr. Crawley described how IL-2, IL-4, IL-15, and IL-21 decrease CD127 expression on isolated $CD8^+$ T cells. Similar to IL-7, IL-4, and IL-15 induced CD127 secretion, as detected by a CD127-specific ELISA. The biological significance of soluble/circulating CD127 *in vivo*, its relevance in HIV infection and contribution to impaired IL-7 bioactivity remains to be investigated. Given that IL-7 resulted in the release of CD127 from $CD8^+$ T cells *in vitro* and that increased plasma IL-7 is associated with both HIV-induced lymphopenia [107] and decreased CD127 expression in HIV infection, we investigated whether

CD127 could be found in human plasma in health and HIV disease. This study reports for the first time that CD127 can be found in human plasma. Moreover, we have documented the novel observation of an increase in plasma CD127 in HIV-infected individuals compared to uninfected individuals (FIGURE 17B). Similar to the activity of other secreted cytokine receptors, such as the receptors for TNF-α and IL-6 which may have immunoregulatory functions [135], and a recent identification of a single nucleotide polymorphism in the IL-7Rα gene, influencing the amount of soluble and membrane-bound isoforms of the protein by putatively disrupting an exonic splicing silencer [136], it is possible that IL-7-induces secretion of CD127. Any soluble CD127 would bind circulating IL-7, resulting in decreased IL-7 bioavailability and alter the dynamics of the IL-7 signalling network in $CD8^+$ T cells as well as multiple other cell types expressing CD127.

Expression of CD127 on both naïve and memory $CD8^+$ and $CD4^+$ T cells has recently been inversely correlated with $CD8^+$ T cell exhaustion in the context of persistent exposure to antigen resulting in the deletion of antigen-specific naïve or memory cells [86, 137]. Both latent Epstein-Barr Virus (EBV) and Cytomegalovirus (CMV), and chronic HIV and Hepatitis C virus (HCV) viral infections have been associated with CD127 down-regulation, the implications of which have yet to be fully described [75, 86, 138]. The cellular mechanism linking antigen persistence to decreased CD127 expression is not known, however, the expression of CD127 on CMV-specific $CD8^+$ T cell subsets appears to confer antigen-independent proliferation in response to IL-7 in contrast to the

proliferation of CD127- cells which required antigen and CD4 T cell help or other cytokines [139]. These data directly demonstrates that IL-7 down-regulates CD127 and suggest a biological consequence that may explain reported correlations of increased plasma IL-7 and decreased CD127 in HIV patients [115, 130]. Furthermore, the loss of CD127 expression has been correlated with the expansion of effector $CD8^+$ T cell with cytolytic activity during chronic HIV infection [85, 138]. Therefore, it appears that antigen persistence either via or in addition to cytokine control are mechanisms for the down-regulation of CD127 *in vivo*. The recent observation that HIV tat, to the exclusion of other HIV and non-HIV proteins, decreases CD127 on $CD8^+$ T cells [140] suggests a degree of pathogen specificity and that CD127 down-regulation is not simply a non-specific response to immune activation.

These results support a role for IL-7 in the down-regulation of CD127 expression and impairment of CTL function observed in HIV infection. A consequence of down-regulating CD127 expression in states of chronic immune activation or persistent infection may be impaired CTL activity and memory cell development. As an increase in IL-7 occurs in the face of declining CTL activity, providing IL-7 therapeutically is unlikely to enhance HIV-specific cellular immunity, as hypothesized by others [125]. This is supported by the observation that although therapeutic use of IL-7 in SIV-infected rhesus macaques resulted in an augmentation of CD4 counts following anti retroviral treatment, it had no effect on the control of viral replication [89]. Alternatively, enhancing IL-7 signalling by increasing the expression of, or enhancing the function of CD127 may result in

improved $CD8^+$ T cell function and enhanced control of viral replication. To this end, identifying the alteration of CD127 expression and function during HIV infection and determining the regulatory mechanisms may provide insights into the development of novel immune based therapies for patients infected with HIV. Moreover, improved understanding of CD127 regulation may potentially provide valuable insights into normal T cell physiology and the role of this receptor in other diseases in which cellular immune function and IL-7 activity may be altered.

4.1.2 Role of Viral Factors in the Down-regulation of CD127 on $CD8^+$ T cells

Direct infection of human $CD8^+$ T cells by HIV has recently been described in an *in vitro* model [141] but appears to be a very rare event *in vivo* and does not likely explain the decreases in CD127 expression. The activity of HIV proteins with immunoregulatory activity, such as gp120 [142], may contribute to the decrease in CD127 expression on $CD8^+$ T cells, however, HIV-1 gp120 or HIV-1 nef did not alter CD127 expression on $CD8^+$ T cells.

Here, soluble HIV-1 tat down-regulated surface CD127 expression on purified $CD8^+$ T cells isolated from healthy individuals compared to cells maintained in medium alone. The effect was dose and time dependent and did not affect viability of the cells. Faller *et al.* have confirmed these findings and have demonstrated that this down-regulation results in impaired $CD8^+$ T cell proliferation and perforin synthesis after IL-7 stimulation [140]. HIV-1 tat protein may in part be responsible for the down-regulation of CD127 on the surface of $CD8^+$ T cells. In our experiments, the concentration of HIV-1 tat used was significantly higher than estimates of HIV-1 tat concentration in patient sera (300-

500 ng/ml) [143]. However, it has been suggested by Faller *et al.* that the commercially available HIV-1 tat used is grown in bacteria and may not be acetylated at Lys28 or Lys50, which would significantly enhance the activity of HIV-1 tat protein [140]. The effect of HIV-1 tat protein on CD127 gene expression was studied to further evaluate the mechanism of CD127 down-regulation on $CD8^+$ T cells. Although HIV-1 tat protein decreased cell surface CD127 expression, it did not alter CD127 gene expression, suggesting that an alternate mechanism, either receptor internalization or receptor shedding, is responsible for this observed down-regulation in CD127 on $CD8^+$ T cells. These are currently being evaluated.

4.2 To determine if *in vitro* HIV down-regulates CD127 expression on $CD8^+$ T cells

Since *in vivo* HIV infection results in a decrease in surface CD127 expression on circulating $CD8^+$ T cells [83], the role of *in vitro* HIV-1 on surface CD127 expression was studied. HIV infection involves docking of the virus to its target cells, and binding of gp120 to chemokine receptors on the surface of the target cells. HIV infections are usually initiated by virions that use the CCR5 chemokine receptors (R5 tropic) and replicate in memory T cells concentrated in mucosal tissues as well as monocytes. Viruses using CXCR4 chemokine receptors (X4 tropic) replicate in thymocytes and naïve T cells and are more common in late disease [144]. In addition, infection with HIV has been demonstrated to stimulate the production of chemokines and cytokines from a variety of cell types [145]. Both cytokines and chemokines modulate CCR5 and

CXCR4 availability, resulting in differential replication potentials for R5 and X4 HIV strains [3]. To determine if R5 or X4 HIV differ in their effect on CD127 expression, which may explain differences in pathogenicity, the influence of an R5 tropic HIV-1_{Ba-L}, the X4 tropic HIV-1_{IIIB} and the dual tropic HIV-1_{CS204} strain of HIV were studied. Infection of PBMCs with each of the strains of HIV-1 resulted in a transient decrease in surface CD127 expression on $CD8^+$ T cells. Culture of purified $CD8^+$ T cells with each strain of HIV-1 as well as the infection of PBMCs with the replication incompetent HIV-1 from supernatants from 8e5 cells resulted in no change in surface CD127 expression, suggesting that a productive infection is necessary to induce the change in CD127 expression.

Since *in vitro* HIV infection affects CD127 surface expression on $CD8^+$ T cells within PBMC cultures but not in purified $CD8^+$ T cells alone, the possibility of a factor secreted by PBMCs and affecting CD127 expression was considered. Results from experiment involving co-culturing purified $CD8^+$ T cells via a transwell with infected PBMCs or culturing $CD8^+$ T cells directly in supernatants from infected PBMCs, each demonstrated a decrease in surface CD127 expression on $CD8^+$ T cells, suggesting that changes in CD127 on $CD8^+$ T cells is due to the activity of soluble factor(s) present in HIV infected PBMC cultures.

In vitro HIV infection causes production of various cytokines. Because the observed decrease in surface CD127 by in vitro HIV infection is transient and does not alter expression of RNA encoding the transmembrane form of CD127, the mechanism of down-regulation of CD127 may be similar to what was reported in AIM 1, that being receptor shedding. This led us to investigate the

roles of IL-7 and IL-4 in the down-regulation of surface CD127 observed by *in vitro* HIV-1 infection. Quantification of IL-7 and IL-4 by ELISA suggested that the decrease in CD127 expression on $CD8^+$ T cells in PBMC cultures is not due to an increase in IL-7 or IL-4 in supernatants of HIV-1_{CS204} infected PBMCs.

As seen *in vivo*, *in vitro* HIV infection of PBMC results in the down-regulation of CD127 surface expression on $CD8^+$ T cells. This effect appears to be due to the activity of soluble factor(s) present in HIV infected PBMC cultures. Further elucidating the mechanism(s) of CD127 down-regulation will provide important insights into the immunopathogenesis of HIV disease.

4.3 To determine if the functional capacity of CD127 is altered in HIV infection and if this is restored with suppression of viral replication

CD127 down-regulation on $CD8^+$ T cells during HIV infection may contribute to the observed impairment in CTL activity. Enhanced IL-7 production reported in HIV infected individuals may, however, compensate for the decrease in CD127 expression and overcome any immune defects that could be attributed to the number of receptors alone. An alternative explanation for impaired $CD8^+$ T cell activity is a decrease in IL-7 mediated signalling, which would impede the biological function of IL-7 on $CD8^+$ T cells in HIV infection. To address these questions, we examined IL-7 responsiveness of $CD8^+CD127^+$ T cells from untreated HIV-infected individuals and healthy controls. Readouts of IL-7 responsiveness included activation of STAT5, up-regulation of Bcl-2 and cell proliferation and expression of degranulation markers CD107a and CD107b.

This cross-sectional study was comprised of three groups of individuals: 1) untreated viremic HIV-infected individuals, 2) HIV-infected individuals on effective antiretroviral therapy (HAART) and 3) healthy controls. Untreated HIV-infected individuals were naïve to any therapy or off therapy for at least one year, and had CD4 counts ranging from 120 to 611 cells/mm^3 and VL of <50 to 365 301 copies/ul. HAART treated patients have been on therapy for at least a year, and had CD4 counts > 400 cells/mm^3 and undetectable viral loads (VL<50 copies/ul). Analysis of whole blood samples from all patients demonstrated that, as expected, untreated HIV-infected individuals had decreased surface CD127 expression on the $CD8^+$ T cells when compared to healthy controls. Patients undergoing effective antiretroviral therapy had a greater proportion of $CD8^+$ T cells expressing CD127 when compared to untreated HIV infected individuals. These results are consistent with previous reports from our laboratory and in the literature [83] and confirm that CD127 expression is decreased in the course of HIV infection.

4.3.1 Activation of STAT5

The JAK/STAT signalling pathway represents a major signal transduction pathway involved in cytokine responses including IL-7. Interaction of IL-7 with its receptor triggers within minutes the JAK/STAT signal transduction cascade, involving the phosphorylation of JAK-1 and JAK-3, kinases that are constitutively associated with the CD127 and γc receptor subunits, respectively. This is followed by STAT5 recruitment to the phosphorylated receptor complex, STAT5

phosphorylation, dimerization, nuclear translocation, and transactivation of target genes, making STAT5 an attractive molecule to study.

Intracellular flow cytometric analysis showed that, in response to IL-7, phosphorylation and activation of STAT5 in $CD8^+CD127^+$ T cells from untreated HIV-infected individuals are significantly decreased compared to healthy controls. In support of these findings, Benoit *et al* have also demonstrated that IL-7 stimulation failed to activate STAT5 in a substantial proportion of $CD8^+$ T cells from HIV-infected individuals [146], suggesting that defects in the IL-7/IL-7 receptor signalling occur in HIV infection. Defects in IL-7 receptor signalling have also been demonstrated in other disease states such as breast cancer, where it was reported that the percentage of CD127 expressing T cells is reduced in the $CD4^+$ T cell population, as well as in a subpopulation of memory $CD8\alpha\alpha^+$ T cells in patients with breast cancer. PBMCs obtained from 13/19 patient with breast cancer did not respond to stimulation with IL-7 as defined by STAT5 phosphorylation, despite expression of CD127 [76].

In addition to IL-7/IL-7 receptor signalling, the JAK/STAT pathway represents a major signal transduction pathway involved in many other cytokine responses and dysfunctions in the JAK/STAT pathway have been confirmed in these other cytokine responses. $CD4^+$ T cells taken from HIV-infected individuals have been shown to respond abnormaly to IL-2 [147]. Subsequently, Kryworuchko et al. attributed these defects to dysfunctions in the JAK/STAT pathway [148], suggesting that deficiencies in IL-7-dependent responses in HIV-

infected individuals are possible and may be due to defects in the JAK/STAT pathway.

When studying patients on effective antiretroviral therapy, $CD8^+CD127^+$ T cells also demonstrated a significant decrease in phosphorylated STAT5 expression following IL-7 stimulation compared to healthy controls. However, although not statistically significant, the mean levels of phosphorylated STAT5 in patients on effective therapy were greater than the mean in untreated HIV-infected individuals. This partial restoration of IL-7 responses at the level of STAT5 activation may illustrate the beneficial effect of antiretroviral therapy and it has been suggested that, through the control of virus replication, HAART interrupts the chronic antigenic activation of the immune system, which would allow recovery of IL-7 responsiveness [148].

The reason for impaired STAT5 activation in HIV infected individuals and its outcome is unknown. Work by Kryworuchko *et al.* has demonstrated that treatment of $CD4^+$ T cells with HIV-1 env protein or surface ligation of CD4 with anti-CD4 monoclonal antibodies inhibited the IL-2-induced activation of JAK-1 and JAK-3, as well as their targets, STAT5a and STAT5b [149]. This suggests that HIV itself or a viral protein may be responsible for this impaired signalling. It has also been suggested that this inadequacy in the JAK/STAT signalling cascade may contribute to the impairment of cytokine mediated $CD4^+$ T cell responses by HIV [149] and may play a role in the loss of CTL responses in HIV infection.

No significant correlation was observed between patient $CD4^+$ T cell counts and phosphorylation of STAT5. Although a trend is present, no significant correlation was observed between levels of CD127 expression and phosphorylation of STAT5, and may be due to the small sample size being analysed. Working on increasing the number of patients in this study and sorting patients out into defined groups depending on their $CD4^+$ T cell counts (CD4 counts of 0-200, 200-400, >400) should be further studied.

4.3.2 Cell Proliferation

It has been well established that T cells from HIV-infected individuals have a lower capacity to proliferate *in vitro* than those from healthy controls. In 2006, Colle *et al* demonstrated that IL-7-dependent proliferation of PBMCs from untreated HIV-infected individuals was lower than in healthy controls [109]. In addition, successful antiretroviral therapy partially restored these IL-7-dependent responses. Here, we also demonstrate altered IL-7-dependent proliferation of $CD8^+CD127^+$ T cells, where $CD8^+CD127^+$ T cells from untreated HIV-infected individuals displayed a reduced amount of IL-7-dependent proliferation than healthy controls.

Initially discovered as a result of its *in vitro* proliferative effect on immature B cells, a good deal of what is known about the activity of IL-7 involves its role in preventing apoptosis and much less is known about its role in cell proliferation. Recently, IL-7 has been recognized as a mediator of homeostasis, controlling the numbers of naïve and memory T cell in the periphery. Since IL-7 has been reported to drive T cell proliferation via STAT5, the reduced proliferation of

$CD8^+CD127^+$ T cells in response to IL-7 may be due to the lower levels of phosphorylated STAT5 observed in HIV-infected individuals. This may be a potential mechanism contributing to the $CD8^+$ T cell impairment seen in HIV disease. Whether activation of STAT5 directly sets off cell cycle progression or whether additional pathways are involved remain to be examined.

Maintenance of $CD8^+$ T cell survival and proliferation is dependent on the ability to acquire sufficient nutrients to support cellular metabolism, and attaining an appropriate cell size is one cellular requirement for progression from G_1 into S-phase. Several groups have demonstrated that IL-7 maintains metabolic activity through the uptake of glucose from the extracellular milieu [150, 151]. The control of cell size via IL-7 has also been shown to depend on PI3K/AKT signalling, resulting in the regulation of expression as well as activity of the glucose transporter GLU1 and the transferin receptor CD71 [152]. Defects in IL-7 signalling may weaken a cells ability to nourish and therefore hinder cell proliferation. Since STAT5 is responsible for initiating the PI3K/AKT signalling cascade during IL-7/IL-7 receptor signalling, it is possible that the decreased levels of phosphorylated STAT5 detected in untreated HIV-infected individuals also affects the cell's capacity to acquire nutrients and attain an appropriate cells size, thereby impeding proliferation.

Studies of IL-7 dose responses have identified a survival function for IL-7 from a proliferative activity, where low doses of IL-7 (< 1 ng/ml) sustained only cell survival and high doses of IL-7 (>1 ng/ml) promoted both cell survival and cycling [37, 153]. In AIM 1, it was demonstrated that IL-7 down-regulates the

level of CD127 expression on $CD8^+$ T cells, however the various concentrations of IL-7, affected surface receptor expression in different ways. Lower concentrations of IL-7 (0-1000 pg/ml), which have been shown to maintain cell survival, did not affect CD127 expression, or only transiently decreased receptor expression. High concentrations of IL-7 (10 000 pg/ml), which have been shown to promote both cell survival and cell proliferation, caused a sustained decrease in levels of CD127. This complex nature of IL-7/IL-7 receptor signalling for proliferation remains to be further clarified, but may involve various components of the cell cycle machinery. Work done in various cell lines as well as in mice has demonstrated that IL-7 may promote T cell proliferation via two regulatory factors: the cdk activating phosphatase Cdc25A and the cdk inhibitor $p27^{kip1}$, and it has been suggested that an alternate mechanism by which IL-7 can promote cell proliferation is through the regulation of proteins that are involved in the G_1/S cell cycle transition [37, 150, 154].

4.3.3 Expression of Bcl-2

In normal T cell development, IL-7 acts as an anti-apoptotic factor by up-regulating Bcl-2 expression and the homeostatic survival effect has been attributed to an antiapoptotic function of IL-7. IL-7 has been shown to regulated various members of the Bcl-2 family, including synthesis of Bcl-2, phosphorylation of Bad and cytosolic retention of Bax [34]. We examined the IL-7-induced up-regulation of Bcl-2 in $CD8^+CD127^+$ T cells from untreated HIV-infected individuals and of healthy controls. In contrast to what was shown by Cole *et al*, where the IL-7-induced overexpression of the antiapoptotic molecule

Bcl-2 was dramatically altered in viremic patients [155], no significant changes is Bcl-2 expression following IL-7 stimulation were detected. Baseline spontaneous Bcl-2 expression in $CD8^+CD127^+$ T cells from both untreated HIV-infected and healthy individuals were similar and no significant differences were seen in the induction of Bcl-2 by IL-7 between these groups. In their cross-sectional study, Colle *et al.* showed significantly lower baseline Bcl-2 expression in untreated viremic patients compared to healthy controls as well as very weak induction of Bcl-2 by IL-7 in these patients. These differences may be explained by the fact that they looked at total $CD8^+$ T cell population where we sorted and examined specifically in $CD8^+CD127^+$ T cells. Some HIV-infected individuals have a much smaller proportion of $CD127^+$ cells in their total $CD8^+$ T cell pool, and changes seen by Colle *et al.* may be due to a smaller number of cells responding to IL-7 and producing Bcl-2. They also demonstrated that highly active antiretroviral therapy partially restored these defects in patients with an undetectable viral load and CD4 counts > 400 cells/mm^3. Patients on effective antiretroviral therapy have greater proportion of $CD8^+$ T cells expressing CD127 therefore this may explain why HAART restores these defects. In addition, Cole *et al.* only saw significant changes in Bcl-2 expression by IL-7 on day 7 of stimulation. In this report, Bcl-2 expression levels were analysed on day 2 of stimulation with IL-7, and are similar to what Colle *et al.* reported on day 3. Vingerhoets *et al.* have also recently demonstrated a decreased sensitivity of T cells to IL-7 in HIV infection, where the inhibitory effects of IL-7 on spontaneous apoptosis in $CD8^+$ T cells from HIV^+ patients was less pronounced than in healthy controls [156]. Likewise to what

was reported by Cole *et al.*, Vingerhoets *et al.* studied the effects of IL-7 in the total CD8 T cell population following 6 day incubation, and have stated that impaired activation by IL-7 in $CD8^+$ T cells from HIV^+ patients corresponded to a lower CD127 expression in these cells.

The reduction or lack of IL-7-dependent activation of STAT5 may have a significant negative impact on the maintenance of $CD8^+$ T cell responsiveness in HIV infection. The activation of STAT5 through JAK3 has been demonstrated to be implicated in the IL-7-dependent synthesis of Bcl-2 [151]. In the untreated HIV-infected individuals, a decreased in the amount of phosphorylated STAT5 following IL-7 stimulation was detected, however, there was no difference in the amount of Bcl-2 synthesized following IL-7 stimulation. Since a degree of STAT5 activation does occur in $CD8^+CD127^+$ T cells from HIV-infected individuals, a threshold level of phosphorylated STAT5 required to initiate Bcl-2 production may be reached, and can explain this observation. Another justification for this observation may be explained by findings by Barata *et al.*, where PI3K activation was shown to be mandatory for IL-7-mediated Bcl-2 up-regulation [152], suggesting that molecules in addition to STAT5 are required. Phosphorylation of STAT5 may not be the limiting factor. Further studies into the signalling pathways initiated by IL-7 and the IL-7 receptor as well as their outcomes would be of great interest.

4.3.4 Expression of CD107a/b

Cytotoxic T cells produce large quantities of IFN-γ and cytotoxic molecules e.g. perforin and granzyme when responding to antigenic stimulation. Recently, a

novel method for studying the cytotoxic potential of $CD8^+$ T cells has been developed and involves measurement of CD107a and CD107b expression levels. Enhanced cytotoxic potential has been demonstrated to correlate with increased expression of CD107a and b on T cells [157]. Given that IL-7 has been demonstrated to increase production of perforin, the role of IL-7 in the cytotoxic potential of $CD8^+CD127^+$ T cells was studied. Flow cytometric analysis showed that $CD8^+CD127^+$ T cell from untreated HIV-infected individuals express slightly higher amounts of CD107a and CD107b. However, IL-7 alone did not significantly affect CD107a and CD107b expression levels on $CD8^+CD127^+$ T cells in either untreated HIV-infected individuals or healthy controls.

Degranulation of activated $CD8^+$ T cells occurs rapidly after TCR stimulation, as a result of the polarized mobilization of microtubules that transport lytic granules towards the immunological synapse formed between the CTL and target cells [8]. Hence, IL-7 alone may not affect degranulation potential, and the effect of IL-7 on $CD8^+CD127^+$ T cell degranulation should likely be studied where TCR cross-linking can occur. Recently, it has been demonstrated that treatment of PBMCs from HIV-infected patient with IL-15 resulted in induction of perforin in association with lymphocyte proliferation and augmentation of TCR-induced degranulation. Further evaluations of the $CD8^+CD127^+$ T cells potential to degranulate following IL-7 stimulation in a milieu where TCR cross-linking can occur is of interest, and are currently being studied in our laboratory.

5 CONCLUSION

Despite its importance, the reason for impaired CTL activity during HIV disease remains elusive. It is possible that decreased CD127 expression is a major reason for the immune dysfunction and, if so, determining the mechanism(s) of its down-regulation is of utmost importance. Overall, this body of work makes a significant contribution to our understanding of the IL-7/IL-7R system and the mechanism of HIV-immunosupression of $CD8^+$ T cells.

Of all the potential viral (HIV-1 gp120, nef and tat) and host (IL-7, IL-4, IL-1-β, IL-6, IL-10, IL-13, TGF-β and TNF-α) factors studied, only IL-7, IL-4 and HIV-1 tat down-regulated CD127 expression. These results support a role for IL-7 and IL-4 in the down-regulation of CD127 expression and impaired CTL function observed in HIV infection and may be mechanisms by which the virus can escape immune-mediated cell killing, and lead to disease progression. How these results translate into the mechanism of CD127 down-regulation *in vivo* remains to be established. As an increase of IL-7 occurs in the face of declining CTL activity, providing IL-7 therapeutically is unlikely to enhance HIV-specific cellular immunity, as hypothesized previously [125]. Alternatively, enhancing IL-7 signalling by increasing the expression of, or enhancing the function of CD127 may result in improved $CD8^+$ T cell function and enhanced control of viral replication. To this end, identifying the abnormalities of CD127 expression and function during HIV infection and determining the regulatory mechanisms may provide insights into the development of novel immune based therapies for patients infected with HIV. The importance of understanding the regulation of

CD127 expression should be emphasized for not only its relevance to HIV therapies but also its potential to provide valuable insights into normal T cell physiology and the role of this receptor in other diseases in which cellular immune function and IL-7 activity may be altered.

Recently, IL-7 has become a candidate to be used as an immunotherapeutic and its clinical development is ongoing. However, when considering the therapeutic potential of IL-7, our findings indicate that IL-7 receptor function in addition to CD127 expression levels must be considered. In 2006, Colle et al. reported dysfunctionalities in the CD4 and CD8 lymphocytes of HIV-infected patients and suggested that abnormal activation of the immune system in HIV-infected patients may be the cause, contributing to the reduced CD4 count and the altered function of the CD8 compartment [109]. In $CD4^+$ T cells, they demonstrated that the significant positive correlation between CD127 expression and IL-7-dependent Bcl-2 up-regulation in healthy individuals is lost in both untreated and treated HIV-infected individuals patients [155], further suggesting that defects within the IL-7/IL-7R signalling pathway (downstream of the IL-7R) may contribute to a deacrese in IL-7 responsiveness [155]. Recently, Kiazyk *et al.* demonstrated that immune activation leads to the down-regulation of CD127, particularly among $CD4^+$ TCM cells, compromising the homeostatic capacity of the T cell pool and eventually leading to immune deficiency associated with HIV disease [158].

Our data collected from HIV-infected untreated individuals also demonstrates a dysfunction in the IL-7/IL-7 receptor system that may be

explained by IL-7-specific signal transduction defects. In sorted $CD8^+CD127^+$ T cells, where CD127 expression is preserved, T cell from untreated HIV-infected individuals are less capable of responding to IL-7 compared to healthy controls. Taken together, our results provide further insights into the mechanism by which CTL function becomes disrupted during HIV infection. The data obtained from untreated viremic patients suggests cellular defects involving parts of the IL-7R signal transduction pathway that may contribute to a decrease in IL-7 responsiveness. When considering IL-7 as a therapeutic, patients naïve to therapy may not benefit as much from IL-7 based therapy alone because aspects of their IL-7/IL-7 receptor signalling system remain dysfunctional. Patients on therapy may benefit more from IL-7 based therapy since their IL-7/IL-7 receptor signalling system is somewhat restored.

As the IL-7/IL-7R system and its disruption during HIV infection continue to be intensively studied, one will get a better understanding of HIV immunopathogenesis and the potential to lead to the development of novel immune based therapies will come about.

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7 CURRICULUM VITAE

PERSONAL INFORMATION

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Social Insurance:	Available upon request
Citizenship:	Canadian
Date of Birth:	1978-08-17
Language Skills:	Fluent in oral and written English, French, Croatian and Polish

ACADEMIC BACKGROUND

September 2000 – 2008:

- Enrolled in the Ph.D. program in the Department of Biochemistry, Microbiology and Immunology at the University of Ottawa, under the supervision of Dr. Jonathan Angel at the Ottawa Health Research Insitute, 501 Smyth Road, Ottawa, Ontario, K1H 8L6
- Successfully completed all coursework, seminar and research requirements for the Ph.D. program, and permission to commence writing the Ph.D. thesis was granted in July 2008

September 1996 – December 1999:

- Successfully completed B.Sc. degree in the Department of Microbiology and Immunology at McGill University, 845 Sherbrooke Street West, Montreal, Quebec, H3A 2T5

Scholarships and Awards:

- Student and Fellow Travel Award from the 16th Conference on Retroviruses and Opportunistic Infections, February 2009
- University of Ottawa, Department of Biochemistry, Microbiology and Immunology Seminar Award (2007)
- Ontario HIV Treatment Network studentship Award: September 2004 – August 2007
- University of Ottawa Excellence Scholarship: September 2004 – August 2007
- Student and Fellow Travel Award from the 12th Conference on Retroviruses and Opportunistic Infections, February 2005
- Ontario HIV Treatment Network Studentship Award: September 2002 – August 2004
- University of Ottawa Excellence Scholarship: September 2002 – August 2007
- Student and Fellow Travel Award from the 10th Conference on Retroviruses and Opportunistic Infections, February 2003
- Student and Fellow Travel Award from the 9th Conference on Retroviruses and Opportunistic Infections, February 2002
- Student and Fellow Travel Award from the 8th Conference on Retroviruses and Opportunistic Infections, February 2001

Professional Development:

- Have given several poster and oral presentations at numerous national and international scientific conferences
- Assisted in the supervision and mentoring of several graduate and Honour's students
- Played a role in writing and submission of numerous operating grants, several of which were successfully awarded to Dr. Jonathan Angel
- Worked as a Teaching Assistant at the University of Ottawa, Department of Chemistry

EMPLOYMENT HISTORY

- **Laboratory Technician: October 2008 – present**
- Ottawa Health Research Institute, Ottawa
- Primary duties consisted of conducting the laboratory experiments for clinical trials
- I hold the position of laboratory radiation safety officer and health and safety officer
- General ordering

- **Teaching Assistant: September 2002 – May 2003**
- University of Ottawa, Department of Chemistry, Ottawa
- My duties include supervising students during the laboratory sessions, explaining the lab (experiments and techniques), answering questions, correcting laboratory reports and exams.

- **Pharmacy Technician Assistant: September 1999 – June 2000**
- Pharmacie Uniprix, Montreal
- Assisted in filling out prescription and managing cash register

- **Fashion Consultant**: June 1998 – December 1998
- Boutique L'Officiel, Groupe San Francisco Inc., Boucherville
- Assisted customers, organized weekly work schedules, as well as managed cash register and daily deposits

- ***Office Clerk: June 1997- September 1997***
- Protestant School Board of Greater Montreal, Montreal
- Reorganized cataloguing system containing confidential student files

CERTIFICATIONS

- WHIMS Certificate
- Radiation Safety Certificate
- Transport of Dangerous Goods Certificate
- Trained and certified to work in a Bio-Containment Level 3 Laboratory

PEER-REVIEWED PUBLICATIONS

- ***Vranjkovic A***, Crawley AM, Gee K, Kumar A, Angel JB. IL-7 decreases IL-7 receptor alpha (CD127) expression and induces the shedding of CD127 by human $CD8^+$ T cells. Int Immunol. 2007 Dec;19(12):1329-39

- ***Vranjkovic A***, Pattey A, Angel JB. December 2008 – Manuscript in progress. Defective interleukin-7-dependent signaling in $CD8^+CD127^+$ T lymphocytes from HIV-positive patients. In preparation

- Crawley AM., ***Vranjkovic A.***, Young C., Angel JB. January 2009 - Manuscript in progress. Interleukin-4 downregulates CD127 expression on human thymocytes and mature $CD8^+$ T-cells

CONFERENCE PRESENTATION

- ***Komsic-Vranjkovic A***, Angel JB (2009) Defective interleukin-7-dependent signaling in $CD8^+CD127^+$ T lymphocytes from HIV-positive patients. 16th Conference on Retroviruses and Opportunistic Infections, Montreal, Quebec, Canada

- ***Komsic-Vranjkovic A***, Crawley AM, Gee K, Kumar A, Angel JB (2007) IL-7 decreases IL-7 receptor alpha (CD127) expression and induces the shedding of CD127 by human $CD8^+$ T-cells. 2007 Ontario HIV Treatment Network Research Conference, Toronto, Ontario, Canada

- ***Komsic-Vranjkovic A***, Crawley AM, Gee K, Kumar A, Angel JB (2007) IL-7 decreases IL-7 receptor alpha (CD127) expression and induces the shedding of CD127 by human $CD8^+$ T-cells. Canadian Association for HIV Research 16th Annual Meeting, Toronto, Ontario, Canada

- ***Komsic-Vranjkovic A***, Faller E, MacPherson P and Angel JB (2006) Differential Regulation of IL-7 Receptor (CD127) on $CD8^+$ T cells: Implications for HIV Immunopathogenesis. Canadian Federation of Biological Societies 50th Annual Meeting, 4th Northern Lights Fall Conference, Ottawa, Ontario, Canada

- ***Komsic-Vranjkovic A***, Faller E, MacPherson P and Angel JB (2006) Differential Regulation of IL-7 Receptor (CD127) on $CD8^+$ T cells: Implications for HIV Immunopathogenesis. 2006 Ontario HIV Treatment Network Research Conference, Toronto, Ontario, Canada

- ***Komsic-Vranjkovic A***, Faller E, MacPherson P and Angel JB (2005) Differential Regulation of IL-7 Receptor (CD127) on $CD8^+$ T cells: Implications for HIV Immunopathogenesis. 12th Conference on Retroviruses and Opportunistic Infections, Boston, Massachusetts, USA

- ***Komsic-Vranjkovic A***, Faller E, MacPherson P and Angel JB (2004) Differential Regulation of IL-7 Receptor (CD127) on $CD8^+$ T cells: Implications for HIV Immunopathogenesis. 2004 Ontario HIV Treatment Network Research Conference, Toronto, Ontario

- **Komsic-Vranjkovic A**, Faller E, MacPherson P and Angel JB (2004) Differential Regulation of IL-7 Receptor (CD127) on $CD8^+$ T cells: Implications for HIV Immunopathogenesis. Canadian Association for HIV Research 13th Annual Meeting, Montreal, Quebec, Canada

- **Komsic-Vranjkovic A** and Angel JB (2003) Differential Regulation of IL-7 Receptor (CD127) by TNF-α and IL-7: Implications for HIV

Immunopathogenesis. 2003 Ontario HIV Treatment Network Research Conference, Toronto, Ontario

- **Komsic-Vranjkovic A** and Angel JB (2003) Differential Regulation of IL-7 Receptor (CD127) by TNF-α and IL-7: Implications for HIV Immunopathogenesis. Canadian Association for HIV Research 12th Annual Meeting, Halifax, Nova Scotia, Canada

- **Komsic-Vranjkovic A** and Angel JB (2003) Differential Regulation of IL-7 Receptor (CD127) by TNF-α and IL-7: Implications for HIV Immunopathogenesis. 10th Conference on Retroviruses and Opportunistic Infections, Boston, Massachusetts, USA

- **Komsic A** and Angel JB (2002) Interleukin-7 Receptor (CD127) Expression and RNA Accumulation in $CD8^+$ T cells is Downregulated by TNF-α. 2002 Ontario HIV Treatment Network Research Conference, Toronto, Ontario

- **Komsic A** and Angel JB (2002) Interleukin-7 Receptor (CD127) Expression and RNA Accumulation in $CD8^+$ T cells is Downregulated by TNF-α. Canadian Association for HIV Research 11th Annual Meeting, Winnipeg, Manitoba, Canada

- **Komsic A** and Angel JB (2002) Interleukin-7 Receptor (CD127) Expression and RNA Accumulation in $CD8^+$ T cells is Downregulated by TNF-α. 9th Conference on Retroviruses and Opportunistic Infections, Seattle, Washington, USA

- **Komsic A** and Angel JB (2001) IL-7 Receptor (CD127) Expression on CD8 T-cells is Downregulated by TNF-α but not IL-7, IL-10 or gp120. Canadian Association for HIV Research 10th Annual Meeting, Toronto, Ontario, Canada

- **Komsic A** and Angel JB (2001) IL-7 Receptor (CD127) Expression on CD8 T-cells is Downregulated by TNF-α but not IL-7, IL-10 or gp120. 8th Conference on Retroviruses and Opportunistic Infections, Chicago, Illinois, USA

8 CONTRIBUTIONS OF COLLABORATORS

Agatha Komsic-Vranjkovic conducted the research reported in this thesis, with the following exception. The detection of soluble CD127 in human plasma was performed by Dr. Katrina Gee.